GE Healthcare
Life Sciences

Instructions 71-5000-09 AD **Antibody affinity chromatography**

rProtein A Sepharose™ Fast Flow

Introduction

rProtein A Sepharose Fast Flow is an affinity medium, designed for the purification of monoclonal and polyclonal antibodies at both laboratory and process scale.

The specificity of protein A is primarily for the Fc region of IgG, through which it binds, leaving the antigen binding sites free. However, it can also bind the Fab region through secondary sites. There are differences between the binding affinities for Fc and Fab, usually Fc binding is stronger, which can provide a means of fractionating Fab or $F(ab)_2$ from Fc.

The following pages contain information about media characteristics, column packing, method design, method optimization, scale up and maintenance. Follow the instructions to ensure best performance and trouble-free operation from rProtein A Sepharose Fast Flow.

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1 Characteristics

The recombinant protein A used in the manufacture of rProtein A Sepharose Fast Flow has been specially engineered to give very high binding capacities. It is produced in *E. coli* and purified by a multi-step chromatographic procedure. Purification does not involve the use of IgG or any other proteins. The purified recombinant protein A is tested according to established specifications before being released for the manufacture of rProtein A Sepharose Fast Flow.

The base matrix, Sepharose 4 Fast Flow, is a highly cross-linked, 4% agarose derivative with excellent chemical and physical stabilities, making it ideal for process scale applications.

Purified recombinant protein A is coupled to Sepharose 4 Fast Flow by a technique which generates a stable thioether linkage between protein A and the base matrix. The coupling technique is optimized to give a high binding capacity for IgG. This binding capacity, together with the excellent kinetic and flow properties of the cross-linked, 4% agarose base matrix, permits rapid processing of large volumes of dilute cell culture fluid. The total binding capacity of human IgG is approximately 50 mg/ml drained medium.

The dynamic capacity of chromatographic adsorbents is a function of the flow velocity used and it increases with decreasing flow velocity. An example of the flow velocity/capacity dependence for three different flow velocities is shown in Figure 1. Figure 2 shows typical pressure/flow velocity characteristics.

The high chemical stability of rProtein A Sepharose Fast Flow enables it to withstand rigorous cleaning and sanitizing procedures, despite the relatively labile nature of protein ligands.

The characteristics of rProtein A Sepharose Fast Flow are summarized in Table 1.

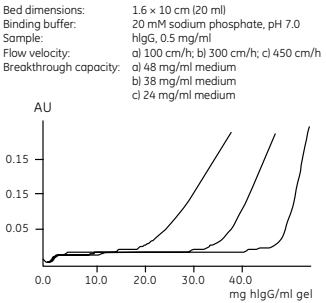

Fig 1. Example of flow velocity/capacity dependence for rProtein A Sepharose Fast Flow. Breakthrough capacity for hIgG was determined at three different flow velocities. Breakthrough capacity is defined as mg hIgG applied per ml gel at the point where the concentration of hIgG in the column effluent reaches a value of 5% of the concentration in the sample.

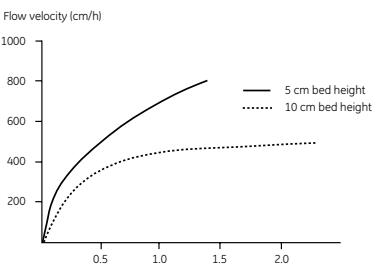

Fig 2. Pressure/flow velocity characteristics of rProtein A Sepharose Fast Flow. The pressure/flow velocity data was determined in a BPG™ 200/500 column (200 mm i.d.) packed to a bed height of 5 cm and 10 cm using water as the mobile phase at 20°C.

Table 1. Characteristics of rProtein A Sepharose Fast Flow

Composition	highly cross-linked 4% agarose
Particle size	60 to 165 µm
Ligand	recombinant protein A (*E. coli*)
Ligand density	approx. 6 mg protein A/ml drained medium
Coupling chemistry	epoxy
Total binding capacity	approx. 50 mg human IgG/ml drained medium
Chemical stability[1]	Stable in all aqueous buffers commonly used in protein A chromatography – 10 mM HCl (pH 2), 1 mM NaOH (pH 11), 0.1 M sodium citrate/HCl (pH 3), 6 M guanidine-HCl, 20% ethanol
Recommended pH	
Working range	2 to 9[3]
Cleaning-In-Place	2 to 11
Recommended working flow velocity	30 to 300 cm/h
Temperature stability[2]	2°C to 40°C
Delivery conditions	20% ethanol

[1] No significant change in chromatographic performance after either 1 week storage or 100 cycles of normal use, at room temperature.

[2] Recommended long term storage conditions: 2°C to 8°C, 20% ethanol

[3] pH below 3 is sometimes required to elute strongly bound Ig´s. However, protein ligands may hydrolyze at very low pH.

2 Packing columns

rProtein A Sepharose Fast Flow is supplied as a suspension in 20% ethanol. Decant the 20% ethanol solution and replace it with packing buffer before use.

2.1 Recommended columns

Lab-scale columns

- Tricorn™ 10/100 (10 mm i.d.) for bed volumes up to 8.5 ml at bed heights up to 11 cm.
- XK 16/20 (16 mm i.d.) for bed volumes up to 30 ml at bed heights up to 15 cm.
- XK 26/20 (26 mm i.d.) for bed volumes up to 80 ml at bed heights up to 15 cm.

Process scale columns

- BPG 100/500 (100 mm i.d.) for bed volumes up to 2.4 litres at a maximum bed height of 30 cm.
- BPG 200/500 (200 mm i.d.) for bed volumes up to 9.4 litres at a maximum bed height of 30 cm.
- BPG 300/500 (300 mm i.d.) for bed volumes up to 21 litres at a maximum bed height of 30 cm.
- BPG 450/500 (450 mm i.d.) for bed volumes up to 43 litres at a maximum bed height of 27 cm.
- Process Stack (PS) 370 (370 mm i.d.) with a bed volume of 16 litres at a fixed bed height of 15 cm.
- INdEX™ 100/500 (100 mm i.d.) for bed volumes up to 2.4 litres at a maximum bed height of 30 cm.
- INdEX 200/500 (200 mm i.d.) for bed volumes up to 9.4 litres at a maximum bed height of 30 cm.
- BioProcess™ Stainless Steel (BPSS) 400/150; 600/150; 800/150; 1000/150; 1200/150 and 1400/150 (4001400 mm i.d.) with fixed bed volumes ranging from 19 to 230 litres at a fixed bed height of 15 cm.

2.2 Packing lab-scale columns

1. Assemble the column (and packing reservoir, if necessary).
2. Remove air from the column dead spaces by flushing the end-piece and adapter with packing buffer. Make sure no air has been trapped under the column net. Close the column outlet leaving the net covered with packing buffer.
3. Resuspend the medium stored in its container by shaking (avoid stirring the sedimented medium). Mix the packing buffer with the medium to form a 50% to 70% slurry (sedimented bed volume/slurry volume = 0.5 to 0.7).
4. Pour the slurry into the column in a single continuous motion. Pouring the slurry down a glass rod held against the column wall will minimize the introduction of air bubbles.
5. If using a packing reservoir, immediately fill the remainder of the column and reservoir with packing buffer. Mount the adapter or lid of the packing reservoir and connect the column to a pump. Avoid trapping air bubbles under the adapter or in the inlet tubing.
6. Open the bottom outlet of the column and set the pump to run at the desired flow velocity. Ideally, rProtein A Sepharose Fast Flow is packed at a constant pressure of approximately 1 bar (0.1 MPa). If the packing equipment does not include a pressure gauge, use a packing flow velocity of approximately 400 cm/h (10 cm bed height, 25°C, low viscosity buffer).

If the recommended pressure or flow velocity can not be obtained, use the maximum flow velocity the pump can deliver. This should also give a reasonable well-packed bed.

Note: *Do not exceed 75% of the packing flow velocity in subsequent chromatographic procedures.*

7. When the bed has stabilized, close the bottom outlet and stop the pump.
8. If using a packing reservoir, disconnect the reservoir and fit the adapter to the column. If using an HR 10/10 column, carefully place the top filter on top of the bed before fitting the adapter.
9. With the adapter inlet disconnected, push the adapter down, approximately 2 mm into the bed, allowing the packing solution to flush the adapter inlet.
10. Connect the pump, open the bottom outlet and continue packing. The bed will be further compressed at this point and a space will be formed between the bed surface and the adapter.
11. Close the bottom outlet. Disconnect the column inlet and lower the adapter approximately 2 mm into the bed. Connect the pump. The column is now ready to use.

2.3 Packing process scale columns

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.

rProtein A Sepharose Fast Flow is easy to pack since its rigidity allows the use of high flow velocities, see Figure 2. Four suitable types of packing methods are described:

- Pressure packing (for columns with adapters)
- 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. Therefore, it is very important to pack and test the column according to the following recommendations.

Begin the packing procedure by determining the optimal packing flow velocity. Guidelines are given for determining the optimal packing flow velocities for columns with adapters and fixed bed heights.

Determining optimal packing flow velocity

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

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

1. Calculate the exact amount of medium needed for the slurry (this is especially important for columns with fixed bed heights). The quantity of medium required per litre packed bed is approximately 1.15 litre sedimented medium.
2. Prepare the column exactly as for column packing.
3. Begin packing the column at a low flow velocity (e.g. 30% of the expected max flow velocity) and record the flow velocity and back pressure when the bed is packed and the pressure has stabilized.
4. Increase the flow velocity in small steps and record the flow velocity and pressure at each step after the pressure has stabilized.
5. Continue recording flow and pressure until the maximum flow velocity has been reached, i.e. when the flow velocity levels off at a plateau indicating bed compression or when the pressure reaches the pressure specification of the column used.
6. Plot pressure against flow velocity as indicated in Figure 2. The optimal packing flow velocity/pressure is 70% to 100% of the maximum flow velocity/pressure.

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

Note: *For BPSS columns, first pack the column by suction packing at a low flow velocity. Then determine the flow/pressure characteristics as above by pumping buffer downwards through the column.*

Pressure packing (BPG columns)

BPG Columns are supplied with a movable adapter. They are packed by conventional pressure packing by pumping the packing solution through the chromatographic bed at a constant flow velocity (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% to 70% slurry. (sedimented bed volume/slurry volume = 0.5 to 0.7). Pour the slurry into the column. Insert the adapter and lower it to the surface of the slurry, making sure no air is trapped under the adapter. Secure the adapter in place.

3. Seal the adapter O-ring and lower the adapter a little into the slurry, enough to fill the adapter inlet with packing solution.
4. Connect a pump and a pressure meter and start packing at the pre-determined packing flow velocity (or pressure). Keep the flow velocity (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 bed has stabilized, close the bottom valve and stop the pump. The bed starts rising in the column. Loosen the O-ring and lower the adapter to 0.5 to 1.0 cm above the bed surface.
6. Seal the O-ring, start the pump and continue packing. Repeat steps 5 and 6 until there is a gap of 1 cm between bed surface and adapter when the bed has stabilized. Mark the bed height on the column tube.
7. Close the bottom valve, stop the pump, disconnect the column inlet and, without loosening the adapter O-ring, push the adapter down to approximately 3 mm below the mark on the column tube. The packing solution will flush the adapter inlet. Remove any trapped air by pumping liquid from the bottom (after the inlet tubing and the bottom valve have been properly filled).

Combined pressure/suction packing (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, to be used as a packing device.
2. 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.
3. Pour the slurry into the column. Add buffer to within 1 to 2 cm of the rim of the upper section. Stir gently to give a homogeneous slurry. Add buffer upto the upper rim and secure the lid in place.
4. Connect a pump and a pressure meter and start packing at the pre-determined packing flow velocity (or pressure). Keep the flow velocity (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 bed 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 Figure 3.

   Packing buffer is now re-circulated in 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 is 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 to 8 mm of the bed surface, close the valve at the 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.

Note: *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.

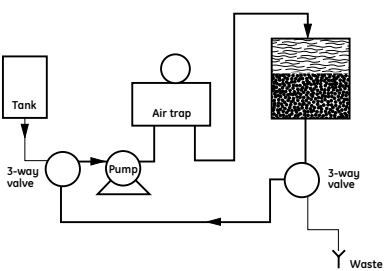

Fig 3. Equipment set up for pressure/suction packing.

Suction packing (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 velocity.

1. Fit a packing device on top of the column tube.
2. Pour some water (or packing buffer) into the column. Make sure that there is no air trapped under the bottom net. Leave about 2 to 3 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 make sure it is homogeneous.

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 pre-determined flow velocity. Keep the flow velocity constant during packing.
5. When the bed has stabilized, the top of the bed should be just below the junction between 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.

Note: *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.

Hydraulic packing (INdEX columns)

INdEX Columns are supplied with a hydraulic function which allows an extremely simple, rapid and reproducible packing procedure. The medium is packed at the same time as the adapter is lowered into position at the correct pressure.

The adapter 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.

When the adapter reaches the surface of the settled medium, it continues downwards under hydraulic pressure compressing the medium. The extent to which the medium is compressed depends upon the pressure from the adapter and the elasticity of the medium. The quantity of medium required when packing rProtein A Sepharose Fast Flow by hydraulic pressure is approximately 1.2 litre sedimented medium per litre packed bed.

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 packing solution up to the top of the glass tube and mix the slurry. Allow the medium to sediment to just below the bevel of the glass tube (G), see Figure 4.
3. Put the adapter in a resting position against the bevel of the glass tube. Avoid trapping air bubbles under the adapter by slightly tilting the adapter while mounting.
4. Lower the lid and secure it in place.
5. Connect a pump to the inlet of the hydraulic chamber (A), 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), and the hydraulic outlet (C). Start the pump and flush the hydraulic chamber (E) free of air and any residual medium.
7. Close (C) and open the elution inlet/outlet (B) to allow trapped air in the adapter net to escape.
8. Close (B) and open the elution inlet/outlet (D) to start the packing, applying a pre-defined constant hydraulic packing pressure. When packing rProtein A Sepharose Fast Flow media in an INdEX column to a bed height of 15 cm, the recommended hydraulic packing pressure is 0.7 bar.
9. When the adapter has reached the surface of the settled bed, continue to run the pump until the adapter has been lowered 5 mm into the packed bed.
10. Close (A) and (D) and stop the pump.
11. Run the column with upward flow for a few minutes to remove residual air trapped in the adapter. The column is now ready for use.
12. To unpack the column, connect the outlet from the pump to (B) and open (C) while keeping (D) closed. This will cause the adapter to rise from the bed surface.

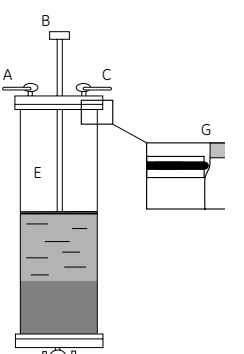

Fig 4. Schematic representation of INdEX column with a 4-port (2-way) valve mounted at the bottom outlet.

3 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 as test substance and 0.5 M NaCl in water as eluent.

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 velocity, 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 velocity 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 velocity:	15 cm/h
UV:	280 nm, 1 cm, 0.1 AU

Calculate HETP and A_s from the UV curve (see example in Fig 5) (or conductivity curve) as follows:

$$HETP = L/N$$

$$N = 5.54(V_e/W_h)^2$$

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 as:

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 to 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.

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

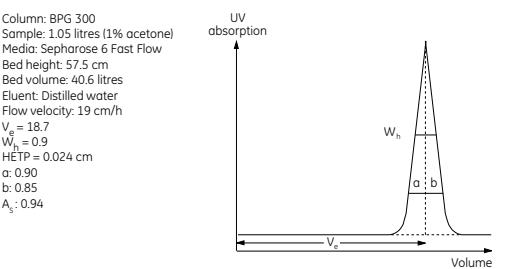

Fig 5. UV trace for acetone in a typical test chromatogram showing the HETP and A_s calculations.

4 Method design and optimization

As with most affinity chromatography media, rProtein A Sepharose Fast Flow offers high selectivity which renders efficiency related parameters such as sample load, flow velocity, bead size and bed height less important for resolution.

The primary aim of method optimization is to establish the conditions that bind the highest amount of target molecule, in the shortest time and with the highest product recovery.

4.1 Specificity and affinity

The degree to which protein A binds to IgG varies with respect to both origin and antibody subclass (Table 2). There might even be a substantial diversity in binding characteristics within a single subclass. This is an important consideration when developing the purification protocol. To achieve efficient Capture of the target antibody, it is often necessary to enhance the binding strength by 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 uninhibited affinity interaction.
- Increasing salt concentration reduces electrostatic repulsion, and increases hydrophobic interactions.
- Reducing temperature has been reported to improve binding.

Table 2. Affinity of protein A for selected classes of monoclonal antibodies. This table is compiled from a variety of sources. Comparisons should be understood to be approximate since they are derived from runs conducted under a variety of conditions.

Antibody	Affinity	Binding pH	Elution pH
Human			
IgG_1	very high	6.0 to 7.0	3.5 to 4.5
IgG_2	very high	6.0 to 7.0	3.5 to 4.5
IgG_3	low-none	8.0 to 9.0	$\leq$ 7.0
IgG_4	low-high	7.0 to 8.0	3.0 to 6.0
Mouse			
IgG_1	low	8.0 to 9.0	4.5 to 6.0
IgG_{2a}	moderate	7.0 to 8.0	3.5 to 5.5
IgG_{2b}	high	$\approx$ 7.0	3.0 to 4.0
IgG_3	low-high	$\approx$ 7.0	3.5 to 5.5

4.2 Method screening

The affinity of rProtein A Sepharose Fast Flow for antibodies of different species, classes and subclasses varies. So the initial screening should be conducted under conditions that bind the largest diversity of antibodies and reveal the relationship between the target antibody and possible contaminating antibodies.

An effective way of mapping antibody behaviour on rProtein A Sepharose Fast Flow is to bind them at high pH and high salt conditions, then elute them in a reducing linear salt/pH gradient.

It is important to make certain that the antibody is stable under the elution conditions. If there is any doubt about this, titrate the antibody fraction to neutrality immediately upon elution in order not to lose biological activity. Another frequent practice to reduce exposure of the antibody to harsh conditions is to reverse the direction of flow during elution. This also elutes the antibody in a more concentrated form.

Recommended screening conditions

Example of suitable buffers:

- Buffer A: 0.05 M boric acid, 4.0 M NaCl, pH 9.0
- Buffer B: 0.05 M sodium phosphate, 0.05 M sodium citrate, 0.3 M NaCl, pH 3.0

Experimental conditions:

- Equilibrate the column with 10 column volumes of buffer A
- Apply a small sample of antibody
- Wash the column with 5 column volumes of buffer A
- Elute the column with a linear gradient of 10 column volumes to 100% buffer B
- Collect fractions into titrating diluent (e.g. 1.0 M Tris-HCl, pH 8.0 so that the diluent volume equals 5% of the programmed fraction volume)
- Regenerate the column with 5 to 10 column volumes of 100% buffer B
- Re-equilibrate the column with buffer A

Conditions can be subsequently modified to provide the best purification performance. Due to the natural diversity of antibodies, binding and elution conditions must be optimized for the antibody to be purified.

The linear gradient used in the initial experiment will reveal the relative binding requirements of the target antibody relative to the contaminating antibodies.

High salt concentration and high pH will often increase dynamic binding capacity, even for antibodies that bind fairly well to protein A. On the other hand, by decreasing salt concentration and/or pH during binding it may be

possible to avoid binding contaminating antibodies. This may also increase dynamic binding capacity since more binding sites will be available for the target antibody. It will also increase selectivity in the system. The balance between selectivity and capacity must be defined with respect to the nature of the feed, i.e., presence of contaminating antibodies and the purity requirement in the eluted product.

With some antibodies, good binding can be achieved without enhancing binding strength. For other antibodies, e.g., mouse IgG_1, it is usually necessary to add up to 4 M NaCl to the binding buffer and feed material to achieve efficient binding.

When optimizing elution conditions, determine highest pH that allows efficient desorption of antibody from the column. This will prevent denaturation of sensitive antibodies due to exposure to low pH values. Step-wise elution (Fig 6) is often preferred in large scale applications since it is technically simpler than elution with continuous gradients. It also allows the target monoclonal antibody to be eluted in a more concentrated form and provides decreased buffer consumption and shorter cycle times.

Linear gradient elution may be feasible for scale up. Its main advantage is that it provides the best and most reproducible fractionation from contaminating antibodies.

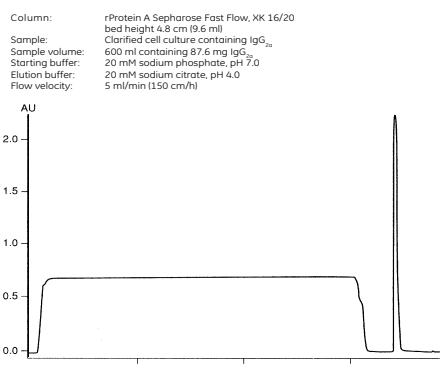

Fig 6. Purification of a monoclonal IgG_{2a} from clarified cell culture on rProtein A Sepharose Fast Flow.

4.3 Optimization of throughput

When optimizing for highest throughput and productivity it is necessary to define the highest sample load over the shortest sample application time with the most acceptable loss in product recovery. The dynamic

binding capacity for the target antibody should be determined by frontal analysis using real process feedstock. Since the dynamic binding capacity is a function of the linear flow velocity applied during sample application, the breakthrough capacity must be defined over a range of different flow velocities.

The optimal flow velocity is that which gives the highest throughput in terms of amount of antibody processed per time unit and volume of media. Example of breakthrough profiles at different flow velocities are shown in Figure 1.

4.4 Removal of leached protein A from final product

Leakage of protein A from rProtein A Sepharose Fast Flow is generally low. However, in many monoclonal applications it is a requirement that leached protein A is eliminated from the final product. There are numerous chromatographic solutions to this problem. Below are suggestions for subsequent chromatographic steps:

- Size exclusion chromatography can be applied for removal of protein A-IgG aggregates by conducting the separation under moderate pH conditions. The large IgG-protein A complexes that are formed will elute early from the column (Fig 7).
- Cation exchange chromatography is an effective tool for removing residual protein A, especially when the particular monoclonal has strong cation exchange binding characteristics. The run is conducted at a pH in which the antibody is known to dissociate from protein A. Protein A binds poorly to cation exchangers and will pass unretained or elute early in the gradient (Fig 8).
- Anion exchange chromatography can also be used to reduce leached protein A contamination. It is best suited to antibodies that are weakly retained on anion exchangers. Because of the strong anion exchange binding characteristics of protein A, protein A-IgG complexes tend to be more strongly retained than non-complex antibodies (Fig 9). These complexes do not generally 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 with 20 mM Tris-HCl, pH 8.5, apply sample and elute in a linear gradient ending at 0.25 M NaCl (20 mM Tris-HCl, pH 8.5). Collect fractions across the antibody peak and screen for protein A.

Column:	HiLoad™ 16/60 Superdex™ 200 pg, bed height 60 cm (120 ml)
Sample:	Purified antibody (14 mg) spiked with recombinant protein A (0.36 mg)
Sample volume:	4.8 ml
Buffer:	20 mM sodium phosphate, 0.15 M NaCl, pH 7.0
Flow rate:	60 cm/h

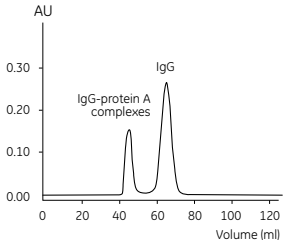

Fig 7. Removal of IgG-protein A complex from mouse IgG_{2a} by size exclusion chromatography on Superdex 200 prep grade. Recombinant protein A was spiked into mouse IgG_{2a} previously purified on rProtein A Sepharose Fast Flow.

Column:	HiTrap™ SP HP (1 ml)
Sample:	Purified antibody (0.61 mg) spiked with recombinant protein A (1.8 mg)
Buffer A:	20 mM sodium citrate, pH 5.2
Buffer B:	20 mM sodium citrate, 1.0 M NaCl, pH 5.2
Flow rate:	150 cm/h
Gradient:	0-45% B, 15 column volumes

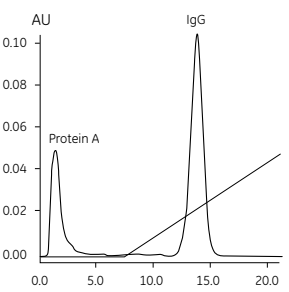

Fig 8. Removal of protein A from mouse IgG_{2b} by cation exchange chromatography on HiTrap SP HP. Recombinant protein A was spiked into mouse IgG_{2b} previously purified on rProtein A Sepharose Fast Flow.

Column:	HiTrap Q HP (1 ml)
Sample:	Purified antibody (0.15 mg) spiked with recombinant protein A (0.009 mg)
Buffer A:	20 mM Tris-HCl, pH 8.5
Buffer B:	20 mM Tris-HCl, 1.0 M NaCl, pH 8.5
Flow rate:	300 cm/h
Gradient:	0-25% B, 20 column volumes

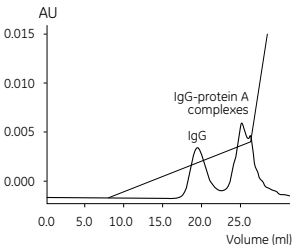

Fig 9. Removal of IgG-protein A complex from mouse IgG_{2a} by anion exchange chromatography on HiTrap Q HP. Recombinant protein A was spiked into IgG_{2a} previously purified on rProtein A Sepharose Fast Flow.

5 Cleaning-In-Place (CIP)

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 velocity.

Regular CIP prevents the build up of these contaminants in the packed bed, and helps to maintain the capacity, flow properties and general performance of rProtein A Sepharose Fast Flow.

CIP protocols

Precipitated or denatured substances	Wash with two column volumes of 6 M guanidine hydrochloride. Wash immediately with at least 5 column volumes of filter-sterilized binding buffer at pH 7 to 8. Use reversed flow direction.
Hydrophobically bound substances	Wash the column with two column volumes of a non-ionic detergent (e.g., conc. 0.1%). Wash immediately with at least five column volumes of filter-sterilized binding buffer at pH 7 to 8. Use Reversed flow direction.
or	Wash the column with three to four column volumes of 70% ethanol. Wash immediately with at least five column volumes of filter-sterilized binding buffer at pH 7 to 8. Use Reversed flow direction. Apply increasing gradients to avoid air bubble formation when using high concentrations of organic solvents.

The CIP protocols should be used as guidelines for formulating a cleaning protocol specific for the feed material applied to the column. The frequency of use will depend on the nature of the feed material, but it is recommended to use a CIP procedure at least every five cycles during normal use. Depending on the nature of the contaminants, different protocols may have to be used in combination. If fouling is severe, the protocols may have to be further optimized.

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 six hours, then wash with at least five column volumes of sterile binding buffer.

or

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

or

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

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

7 Storage

Unused media can be stored in the container at a temperature of 2°C to 8°C. Ensure that the screw top is fully tightened.

Packed columns should be equilibrated in binding buffer containing 20% ethanol to prevent microbial growth.

After storage, equilibrate with at least five bed volumes of starting buffer before use.

8 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.

- Select bed volume according to required binding capacity.
- Select column diameter to obtain a bed height of 5 to 15 cm so that high flow velocities can be used. (See Fig 2, pressure/flow velocity curve. Maximum flow velocity is approximately inversely proportional to the bed height. Expect to operate at no more then 70% of the maximum flow velocity.)
- Define linear flow velocity during sample application to ensure that residence time is not shorter than that established in the small scale experiments. The residence time is equal to the bed height (cm) divided by the linear flow velocity (cm/h) applied during sample loading.
- Keep sample concentration and gradient slope 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.

9 Ordering information

Product	Pack size	Code No
rProtein A Sepharose Fast Flow	5 ml	17-1279-01
	25 ml	17-1279-02
	200 ml	17-1279-03
	1 L	17-1279-04
	5 L	17-1279-05

All bulk media products are supplied in as suspension in 20% ethanol. For additional information, including Data File, Application references and Regulatory Support File, please contact your local GE Healthcare representative.

For local office contact information, visit
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