rProtein A Sepharose Fast Flow

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

- Very high dynamic binding capacity for monocloral antibodies, with high recovery and high purity
- No mammalian components involved during the manufacturing process
- Easy to scale up

rProtein A Sepharose Fast Flow is a high capacity medium for purification of immunoglobulins at lab and process scale.

Characteristics

The recombinant protein A is produced in *E. coli* and has been specially engineered to favour an oriented coupling giving a matrix with enhanced binding capacity. The epoxy based coupling ensures low ligand leakage. The specificity of the recombinant protein A for the Fc region of IgG is similar to native protein A and provides excellent purification in one step. The high capacity, low ligand leakage and a well established base matrix make rProtein A Sepharose Fast Flow ideal for purification of monoclonal antibodies from lab to process scale. The basic characteristics are summarized in Table 1.

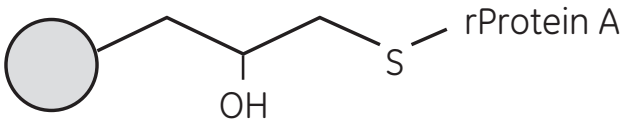

Fig 1. C-terminal cysteine favours oriented thioether coupling.

Enhanced binding capacity due to oriented coupling

The recombinant protein A has been engineered to include a C-terminal cysteine. The epoxy chemistry is controlled to favour a thioether coupling, providing single point attachment of the protein A, see Figure 1. The oriented coupling enhances the binding of IgG. This is illustrated in Figure 2 showing breakthrough curves of human IgG for four different commercially available protein A matrices.

The binding capacity, at 5% breakthrough (10 cm bed height, 190 cm/h flow velocity), was 40 mg hIgG/ml bed volume for rProtein A Sepharose Fast Flow, compared with 27 mg/ml for Competitor A, 24 mg/ml for Protein A Sepharose 4 Fast Flow (native protein A, CNBr coupled) and for Competitor B.

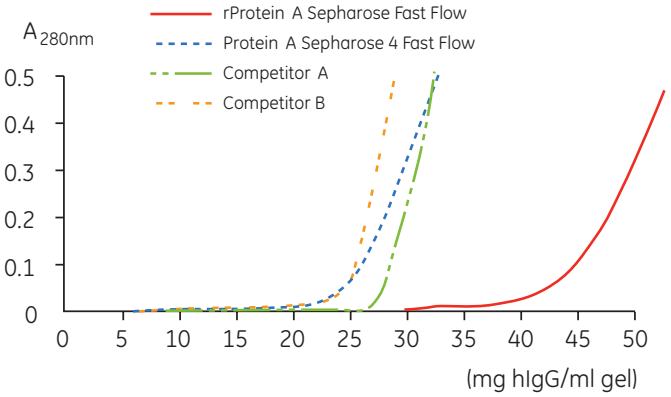

Fig 2. Breakthrough curves of hIgG for four different Protein A media.

Examples of sample loads and recoveries when purifying some monoclonal antibodies are listed in Table 2.

Table 1. Characteristics of rProtein A Sepharose Fast Flow.

Composition	highly cross-linked 4% agarose
Particle size	60–165 µm
Ligand	recombinant protein A (*E. coli*)
Ligand density	approx. 6 mg Protein A/ml medium
Coupling chemistry	epoxy
Binding capacity	
total	approx. 50 mg human IgG/ml medium
dynamic[1]	min 30 mg human IgG/ml medium
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 change in chromatographic 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
Recommended pH	
working range	3–10
cleaning-in-place	2–11
Recommended	
working flow velocity	30–300 cm/h
Temperature stability[2]	4–40 °C
Delivery conditions	20% ethanol

1 Determined at 10% breakthrough by frontal analysis at a linear flow rate of 100 cm/h in a column with a bed height of 5 cm.

2 Recommended long term storage conditions: +4 to +8 °C, 20% ethanol.

Table 2. Purification of some monoclonal antibodies with rProtein A Sepharose Fast Flow.

Monoclonal IgG	Sample load (mg IgG/ml bed volume)	Recovery (%)
IgG_1, mouse[1]	15	83
IgG_{2a}, mouse[1]	11	97
IgG_{2b}, mouse[1]	23	75
IgG_1, humanised[2], *	32	100
IgG_{g2a}, mouse[3], *	8	80

1 Column: XK 16/20, 4.8 cm bed height, 9.6 ml bed volume
Sample: MAb concentrations, 0.2–0.3 mg/ml
Buffer A: 20 mM sodium phosphate, pH 7.0 (+ 3M NaCl for IgG1, mouse)
Buffer B: 0.1 m sodium citrate/NaOH, pH 3.0–4.5
Flow velocity: 150 cm/h

2 Column: 10 mm i.d., 8.2 cm bed height, 6.4 ml bed volume
Sample : MAb concentration 2 mg/ml
Buffer A: 50 mM glycine, 0.25 M NaCl, pH 8.0
Buffer B: 0.1 M glycine, pH 3.5
Flow velocity: 50 cm/h

3 Column: 10 mm i.d., 10 cm bed height, 8 ml bed volume
Sample : MAb concentration 0.1 mg/ml
Buffer A: 50 mM glycine/sodium glycinate, pH 8.8
Buffer B: 50 mM sodium acetate/50 mM sodium citrate, pH 3.5
Flow velocity: 300 cm/h

* Courtesy of Celltech Biologics Plc., UK.

Highly purified recombinant protein A

The recombinant protein A (*E. coli*) is produced in validated fermentation and downstream processes. In addition, no mammalian components are involved in either the fermentation process or the purification of the ligand. The purification process contains several chromatographic steps (but no affinity step with human IgG). Each batch of protein is tested, using validated Quality Control (QC) methods, for IgG binding activity (>95%), electrophoretic purity and reversed phase- (RP-) HPLC purity (>98%), as well as for endotoxin content (<1 EU/mg). Results from QC analysis of five production batches are shown in Table 3.

The recombinant protein A has also been tested and found to have no mitogenic activity in human lymphocytes, in vitro.

Table 3. QC analysis of five production batches of recombinant protein A.

Production batch	IgG binding activity (%)	Purity by RP-HPLC (%)	Endotoxin (EU/mg)
1	97	99.5	<0.1
2	98	99.3	<0.1
3	96	98.9	0.2
4	98	99.6	<0.1
5	96	99.0	0.6

Ligand leakage

Leakage of protein A from rProtein A Sepharose Fast Flow is generally low. The leakage during purification of different IgGs has been analysed using a non-competitive ELISA. The ELISA was developed* to analyse native protein A in the presence of IgG, and has been adapted and evaluated for measurement of this specific recombinant protein A. Typical values found in the IgG containing eluents after purification on rProtein A Sepharose Fast Flow are shown in Table 4. Comparable leakage data from two other media are also included. Protein A Sepharose 4 Fast Flow uses CNBr coupled native protein A. The multi-point attachment achieved with CNBr is slightly more stable, but it also gives less efficient orientation of the protein A and hence lower binding capacity. Another commercially available matrix, Competitor B, was analysed as a reference and showed much higher leakage levels under the same conditions. This matrix uses native protein A, the coupling chemistry is unknown.

In pharmaceutical production processes protein A must be removed from the final product. Leached recombinant protein A can be removed efficiently from the IgG containing fraction using gel filtration or ion exchange chromatography. Figure 3 shows purification of mouse IgG_{2b} on cation exchange after spiking with a large amount of recombinant protein A. Similar results can also be achieved with gel filtration and anion exchange. Methods to remove leached recombinant protein A are described in the Instructions enclosed with each pack of medium.

* The ELISA was developed and adapted to recombinant protein A by Celltech Biologics Plc., UK.

Table 4. Leakage levels of protein A measured with non-competitive ELISA.

Protein A matrix	Protein A conc. in eluate (ng protein A/mg IgG)
rProtein A Sepharose Fast Flow[1]	10–30
rProtein A Sepharose Fast Flow[2]	15
rProtein A Sepharose Fast Flow3	5
Protein A Sepharose 4 Fast Flow[1]	5–10
Competitor B[1]	180–330

1 Polyclonal human IgG, sample load+ 50 mg/ml bed volume elution pH 3.0.
2 Mouse IgG_{2a}, sample load 9 mg/ml bed volume, elution pH 4.0.
3 Mouse IgG_{2b}, sample load 23 mg/ml bed volume, elution pH 3.0.
+ Different levels of breakthrough for the different media (see Figure 2).

Operation

Method development

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

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

Column: HiTrap SP (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 velocity: 300 cm/h
Gradient: 0–45% B, 15 column volumes

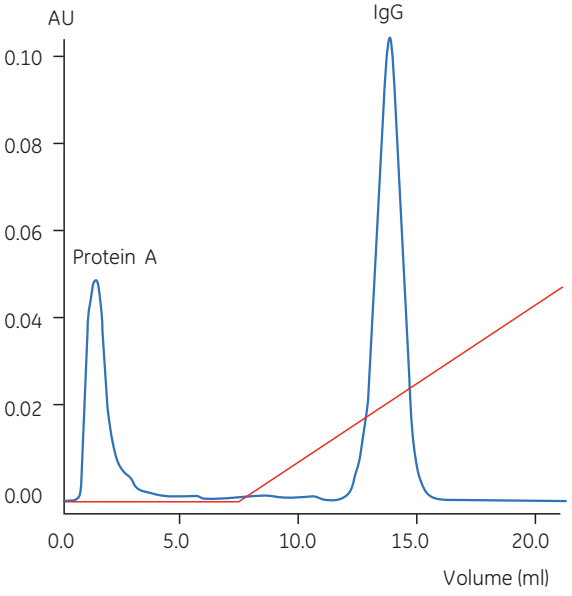

Fig 3. Removal of protein A from mouse IgG_{2b} by cation exchange chromatography on HiTrap™ SP. Recombinant protein A was spiked into mouse IgG_{2b} previously purified on rProtein A Sepharose Fast Flow.

The degree to which protein A binds to IgG varies with respect to both origin and antibody subclass, and even within the same subclass. This is an important consideration when developing the purification protocol.

Typical binding conditions are low salt concentration buffers at neutral pH. To achieve efficient capture of weakly bound antibodies, it is often necessary to increase the pH and/or salt concentration in the binding buffer.

Elution is normally achieved at reduced pH, down to pH 3.5 depending on species and subclass.

Cleaning and sanitization

The general recommendation for cleaning rProtein A Sepharose Fast Flow is to use a mixture of 50 mM NaOH and 1 M NaCl. As an alternative cleaning protocol 6 M guanidine hydrochloride can be used. Phosphoric acid (100 mM) has also been used for cleaning. To remove hydrophobically bound substances a solution of non-ionic detergent or ethanol is recommended.

For sanitizing rProtein A Sepharose Fast Flow, we recommend storage in a solution containing 0.1 M acetic acid/20% ethanol or 2% hibitane digluconate/20% ethanol.

Detailed recommendations for method design and optimization, cleaning and sanitization, and column packing of rProtein A Sepharose Fast Flow can be found in the Instructions that are enclosed with each pack of medium.

Scale up

After optimizing the antibody purification at laboratory scale, the process can be scaled up by keeping the linear flow rate and sample to bed volume ratio constant, and increasing the column diameter. We recommend a bed height of 5–15 cm so that high flow rates can be used. (Pressure/flow velo-city curves for a 20 cm i.d. column are shown in Figure 4.)

Equipment

rProtein A Sepharose Fast Flow can be used together with most equipment available for chromatography from lab scale to production scale. Recommended Amersham Biosciences columns are listed in Table 5.

Application

An example of a purification of monoclonal mouse IgG2a is shown in Fig. 5. IgG2a from clarified hybridoma cell culture was purified on rProtein A Sepharose Fast Flow. The sample load was 9 mg IgG/ml bed volume and the recovery was 95% of highly purified antibody, see SDS PAGE in Figure 6.

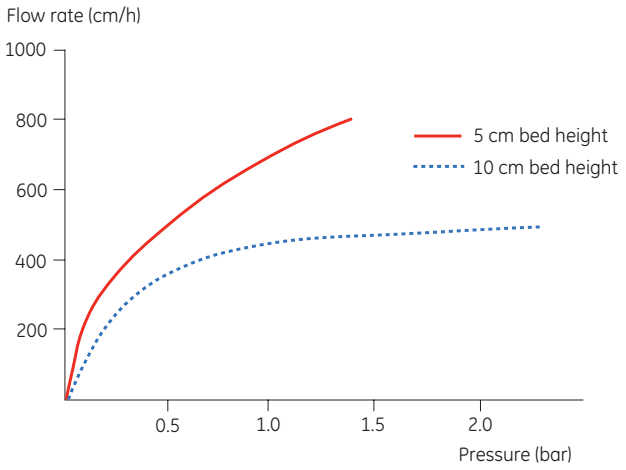

Fig 4. Pressure/flow rate characteristics of rProtein A Sepharose Fast Flow. The pressure/flow velocity data were determined in a BPG 200/500 column (200 mm i.d.) packed to a bed height of 5 cm and 10 cm using water as the mobile phase at 20 °C.

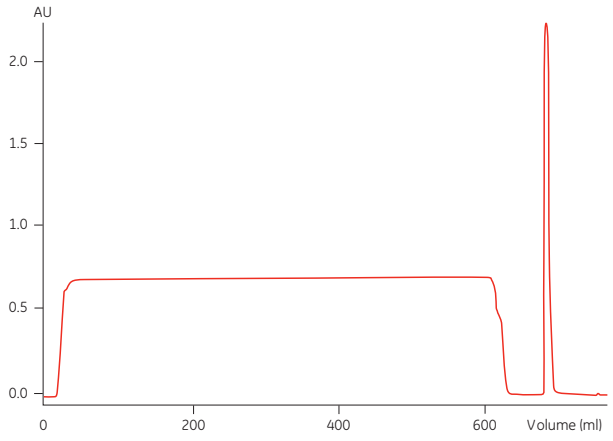

Fig 5. Purification of a monoclonal IgG2a from clarified cell culture on rProtein A Sepharose Fast Flow.

Table 5. Recommended columns for rProtein A Sepharose Fast Flow.

Columns	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 liters	max. 83
BioProcess™ Stainless Steel (BPSS) fixed bed columns	400–1400	12–1500 liters	10–100
INdEX™ variable bed columns	70–200	Up to 24.8 liters	max. 79
Chromaflow™ variable bed columns	280–2000		

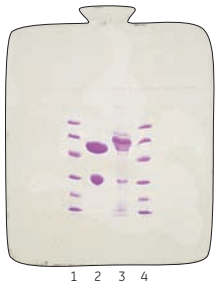

Fig 6. SDS-PAGE of starting material (lane 2) and eluate (lane 3). The samples were concentrated 10 times and reduced. Lane 1 and 4 are LMW markers. PhastSystem™, PhastGel™ Gradient 10–15.

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 suspension in 20% ethanol.

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