rProtein A **Sepharose** Fast Flow

AFFINITY CHROMATOGRAPHY

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

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

Characteristics

The recombinant protein A is produced in *E. coli* and has been specially engineered to favor 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 excellent for purification of monoclonal antibodies from lab to process scale. The basic characteristics are summarized in Table 1.

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 (Fig 2). The oriented coupling enhances the binding of IgG. This is illustrated in Figure 3 showing breakthrough curves of human IgG for rProtein A Sepharose Fast Flow and Protein A Sepharose 4 Fast Flow.

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 and 24 mg/mL for Protein A Sepharose 4 Fast Flow (native protein A, CNBr coupled).

Fig 1. rProtein A Sepharose Fast Flow is a high capacity resin for purification of immunoglobulins at lab and process scale.

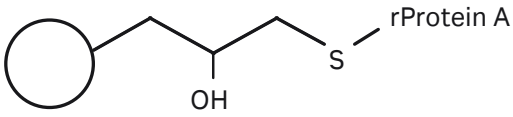

Fig 2. C-terminal cysteine favours oriented thioether coupling.

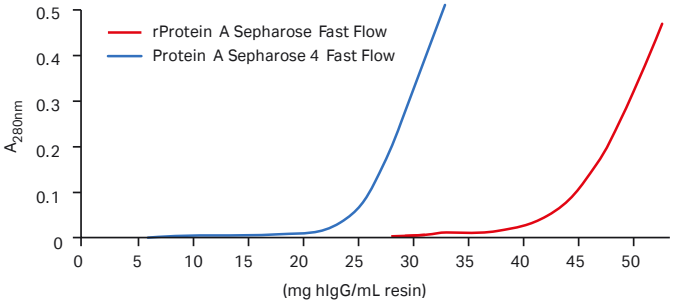

Fig 3. Breakthrough curves of hIgG for rProtein A Sepharose Fast Flow and Protein A Sepharose 4 Fast Flow.

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

Matrix	Cross-linked agarose, 4%, spherical
Particle size, d_{50V} [1]	~ 90 µm
Ligand	Recombinant protein A (*E. coli*)
Coupling chemistry	Epoxy
Dynamic binding capacity, Q_{B10} [2]	~ 35 mg human IgG/mL resin
Chemical stability	Stable to commonly used aqueous buffers, 6 M guanidine hydrochloride, 2% benzyl alcohol, 1 mM NaOH (pH 11), 0.1 M sodium citrate/HCl (pH 3), 20% ethanol
pH stability, operational[3] pH stability, CIP[5]	3 to 10[4] 3 to 12[4,6]
Pressure/flow characteristics	150–250 cm/h at 0.1 MPa in a XK 50/60 column with 5 cm diameter and 25 cm bed height (at 20°C using buffers with the same viscosity as water)[7,8]
Temperature stability	2°C to 40°C
Delivery conditions	20% ethanol
Storage	20% ethanol, 2°C to 8°C

[1] Median particle size of the cumulative volume distribution.

[2] Dynamic binding capacity at 10% breakthrough by frontal analysis at a mobile phase velocity of 100 cm/h in a 7.5/50 PEEK-column at 5 cm bed height (3 min residence time) for human IgG in 0.020 M NaH_2PO_4, pH 7.0

[3] pH range where resin can be operated without significant change in function

[4] pH below 3 is sometimes required to elute strongly bound IgG species. However, protein ligands may hydrolyze at pH below 2.

[5] pH range where resin can be subjected to cleaning-in-place without significant change in function

[6] Reducing agent e.g. 100 mM 1-Thioglycerol followed by 15 mM NaOH is among the most efficient CIP for rProtein A sepharose Fast Flow.

[7] The pressure/flow characteristics describes the relationship between pressure and flow under the set circumstances. The pressure given shall not be taken as the maximum pressure of the resin.

[8] Pressure/flow test performed on the base matrix.

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 another resin is 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.

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 size exclusion chromatography or ion exchange chromatography. Figure 4 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 size exclusion chromatography and anion exchange. Methods to remove leached recombinant protein A are described in the instructions for each resin.

*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 Flow[3]	5
Protein A Sepharose 4 Fast Flow[1]	5–10

[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_{2a}, sample load 23 mg/mL bed volume, elution pH 3.0.
+ Different levels of breakthrough for the different resins (Fig 2).

Operation

Method development

As for most affinity chromatography resins, rProtein A Sepharose Fast Flow offers high selectivity which renders efficiency related parameters such as sample load, flow rate, particle 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 to 45% B, 15 column volumes

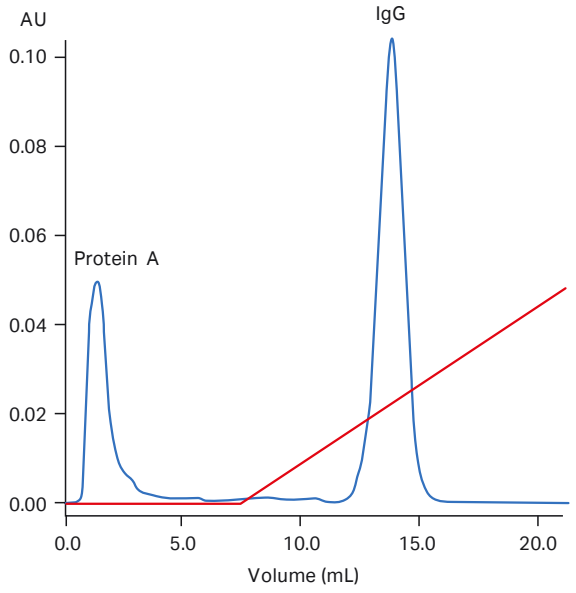

Fig 4. (A) Retention of model proteins (Ribonuclease A and lysozyme) on Capto™ Phenyl (high sub) (green) and Phenyl Sepharose 6 Fast Flow (high sub) (blue). (B) Retention of model proteins (Ribonuclease A and lysozyme) on Capto Butyl (blue) and Butyl Sepharose 4 Fast Flow (green).

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

Reducing agent, for example, 100 mM 1-Thioglycerol followed by 15 mM NaOH is among the most efficient cleaning protocols for rProtein A Sepharose Fast Flow. As an alternative cleaning protocol 6 M guanidine hydrochloride can be used. 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 for each resin.

Scale up

After optimizing the antibody purification at laboratory scale, the process can be scaled up by keeping the flow velocity 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 velocity curves for a 20 cm i.d. column are shown in Figure 5.

Equipment

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

Application

An example of a purification of monoclonal mouse IgG2a is shown in Figure 6. 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 (Fig 7).

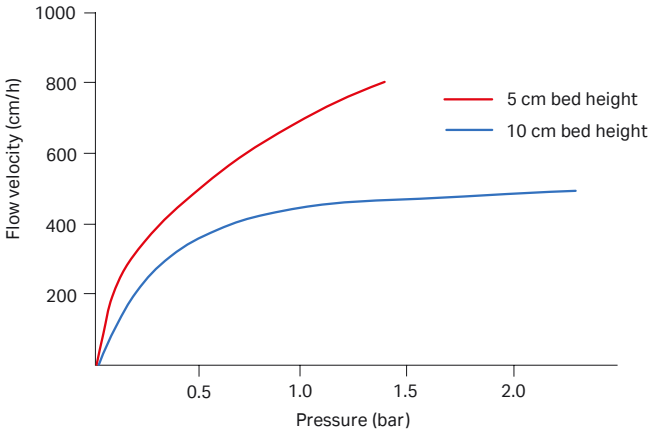

Fig 5. Pressure/flow characteristics of rProtein A Sepharose Fast Flow. The pressure/flow velocity data were determined in a BPG 200/500 column (200 mm i.d.) packed to a bed height of 5 cm and 10 cm using water as the mobile phase at 20°C.

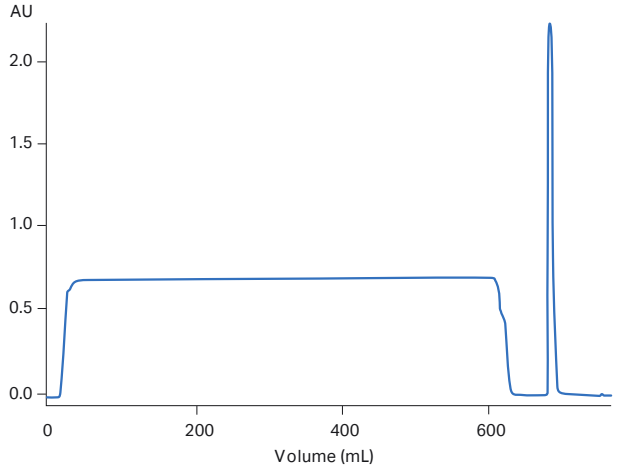

Fig 6. Purification of a monoclonal IgG2a from clarified cell culture on rProtein A Sepharose Fast Flow.

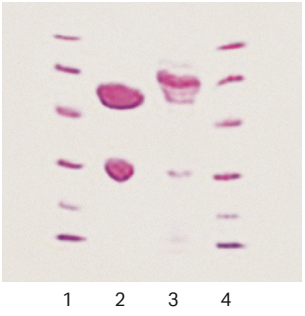

Fig 7. SDS-PAGE of starting material (lane 2) and eluate (lane 3). The samples were concentrated 10 times and reduced. Lane 1 and 4 are LMW markers. PhastSystem™, PhastGel™ Gradient 10–15.

Table 5. Recommended columns for rProtein A Sepharose Fast Flow

Columns	Inner diameter (mm)	Bed volume	Bed height (cm)
Lab scale			
Tricorn™ 10/100	10	up to 3.6–8.4 mL	4.6–10.6
XK 16/20	16	up to 30 mL	up to 15
XK 26/20	26	up to 66 mL	up to 12.5
XK 16/40	16	8–74 mL	max. 35
XK 26/40	26	32–196 mL	max. 35
HiScale™ 16/20	16	up to 40 mL	max. 20
HiScale 16/40	16	16–80 mL	8–40
HiScale 26/20	26	106 mL	max. 20
HiScale 26/40	26	69–212 mL	13–40
HiScale 50/20	50	up to 393 mL	max. 20
HiScale 50/40	50	274–785 mL	14–40
Production scale			
BPG variable bed, glass column	100–450	2.4–131 L	max. 83

Ordering information

Product	Pack size	Code number
rProtein A Sepharose Fast Flow	5 mL	17127901
	25 mL	17127902
	200 mL	17127903
	1 L	17127904
	5 L	17127905
	10 L	17127906
	60 L*	17127907

* Pack sizes available upon request

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

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