Rapid optimisation and development of an automated two-step purification procedure for monoclonal IgG antibodies

Application Note

ÄKTA*FPLC*®

- Convenient and rapid standard purification protocol for monoclonal IgG antibodies in the 1–10 mg range
- The automated two-step purification procedure using affinity and gel filtration chromatography is applicable for a wide range of general purification problems
- Convenient automation of purification procedures
- Rapid and easy optimisation of separation conditions in affinity chromatography using the pre-programmed method templates and scouting functions of ÄKTA*FPLC*

Summary

This application note describes the development and optimisation of an automated two-step purification process for mouse monoclonal IgG antibodies (MAbs) on ÄKTA*FPLC*. Cell culture supernatants containing monoclonal IgG_1 were used as a model system. The purification method consists of a capture step and a polishing step. The antibodies were captured by affinity chromatography on HiTrap rProtein A. Suitable conditions for binding and elution were investigated using the automatic scouting functions available in ÄKTA*FPLC*. The results obtained from the scouting experiments were used as guidelines to develop the final automated purification method. HiLoad 16/60 Superdex 200 prep grade was selected for polishing by gel filtration, allowing trace contaminants to be efficiently removed. All fractions were analysed for purity by SDS-PAGE.

By taking full advantage of system flexibility and software support, the method was then automated, directing the fraction from the affinity column containing the target molecule to the gel filtration column (Figure 1).

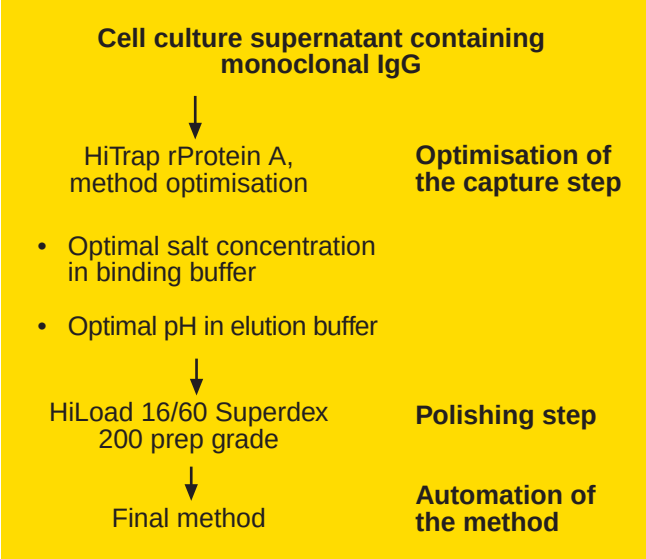

Fig. 1. Overview of the general approach to developing the automated two-step purification method.

The automated method can handle large amounts of sample and provides a convenient way for routine purification of monoclonal IgG antibodies. The strategy using affinity and gel filtration chromatography is, however, applicable to a wide range of purification problems.

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Edition AA

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Introduction

The hybridoma technique for the production of monoclonal antibodies has had a major impact on the life sciences. The increased use of MAbs has created the need for rapid, simple and convenient purification strategies.

In this work we describe in detail the optimisation and development of an automated two-step purification method for monoclonal IgG antibodies based on capture using affinity chromatography and polishing with gel filtration.

The purification of monoclonal IgG_1 from cell culture supernatant was used as a model system. The approach was to take advantage of the flexible design and functions of the system in order to optimise some of the parameters important for the capture step and to create an automated purification process.

Although the method described here was developed for MAbs, the purification strategy is valid for many other affinity applications, including purification of fusion proteins.

Results

Capture

The main purpose of the capture step was to concentrate the target molecule and remove the bulk of the contaminants. Affinity chromatography on HiTrap rProtein A was chosen for its high selectivity for IgG.

Screening for optimal binding conditions

Most mouse monoclonal antibodies of the IgG_1 subclass, in contrast to the other IgG subclasses, require high salt concentrations for binding to immobilised protein A. To establish the appropriate salt concentration for binding of this particular MAb, a scouting experiment was done.

A pre-programmed method template in UNICORN was used for automatic scouting of binding conditions, allowing automatic control of the salt concentration of the binding buffer. A stand-alone pump (Pump P-50) and additional valves were added for automatic sample handling and buffer selection.

Salt concentrations in the range 0–3.5 M sodium chloride in the binding buffer were tested. The salt concentration of the sample was adjusted accordingly.

The scouting runs revealed that 2.5 M NaCl was optimal for binding (Figure 2), and was used during subsequent method optimisation. The best binding conditions were defined as those resulting in largest elution peak areas combined with absence of antibodies in the flow-through.

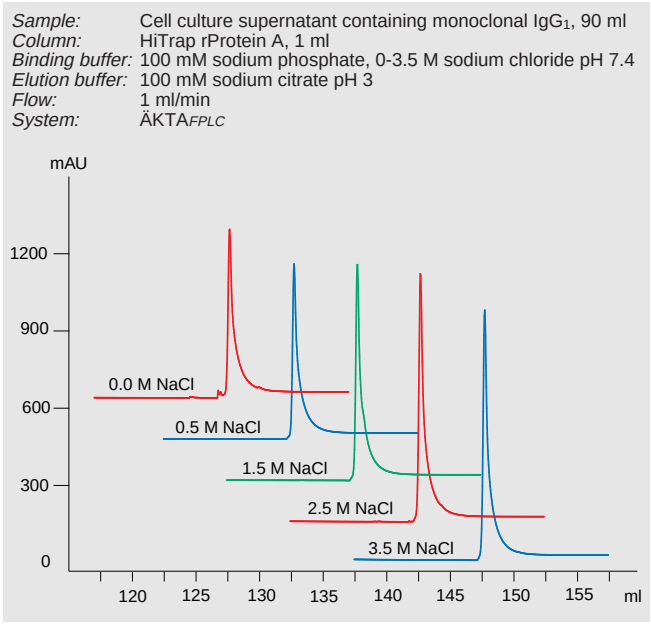


Fig. 2. Automatic scouting of optimal salt concentration in the binding buffer on HiTrap rProtein A.

Screening for optimal elution conditions

In order to determine the optimal pH for elution, a pH flow cell and electrode were connected for in-line monitoring. A stand-alone pump (Pump P-50) and additional valves were added for automatic sample handling and buffer selection.

The optimal pH for elution was determined by eluting the antibodies with a decreasing pH gradient ranging from pH 7.4 to pH 3. Hydrogen phosphate (pKa 7.2) and sodium citrate (pKa 4.76) were chosen as buffer components.

Sample: Cell culture supernatant containing monoclonal IgG_1, 90 ml
Column: HiTrap rProtein A, 1 ml
Binding buffer: 100 mM sodium phosphate, 100 mM sodium citrate, 2.5 M sodium chloride pH 7.4
Elution buffer: 100 mM sodium phosphate, 100 mM sodium citrate, pH-gradient from 7.4 to 3.0
Flow: 1 ml/min
System: ÄKTA*FPLC*

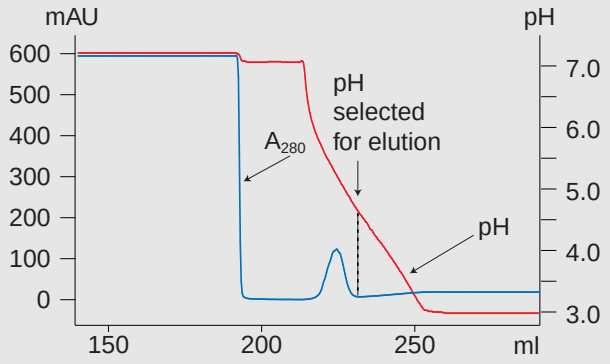


Fig. 3. Automatic scouting of optimal elution pH on HiTrap rProtein A.

The results demonstrated that the peak containing the antibodies was eluted at pH 4.5 (Figure 3). pH 4.5 was therefore selected as elution pH in the final method.

Polishing

The polishing step is the final step in a purification strategy where low or trace levels of contaminants are removed, in this case presumably IgG aggregates and/or dimers. Gel filtration was carried out on HiLoad 16/60 Superdex 200 prep grade column.

Automatic two-step purification

The automated method was based on the results obtained from the previous scouting runs. Automation was achieved by expanding the system with additional valves (Figure 4). The IgG peak from the affinity column was collected in a Superloop. This material was then directed to the gel filtration column. The template used was modified so that the system automatically changed the flow path (based on times from the previous run) and transferred the eluted peak from position 8 on the Outlet Valve through the Injection Valve (injection port, position 3) to a Superloop connected to the injection valve. The eluate from the affinity column is then available for the polishing step. The template for the polishing step

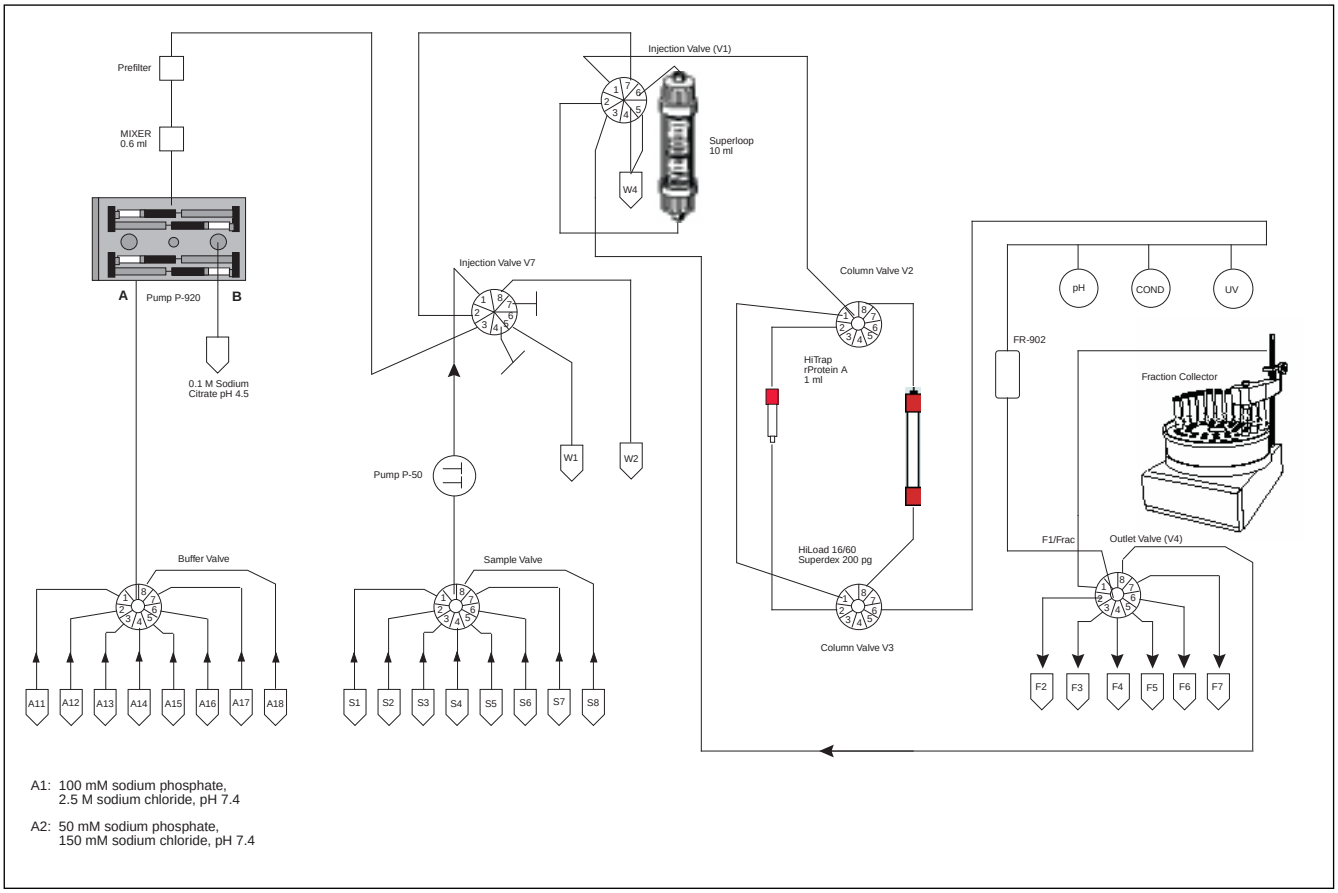


Fig. 4. System configuration.

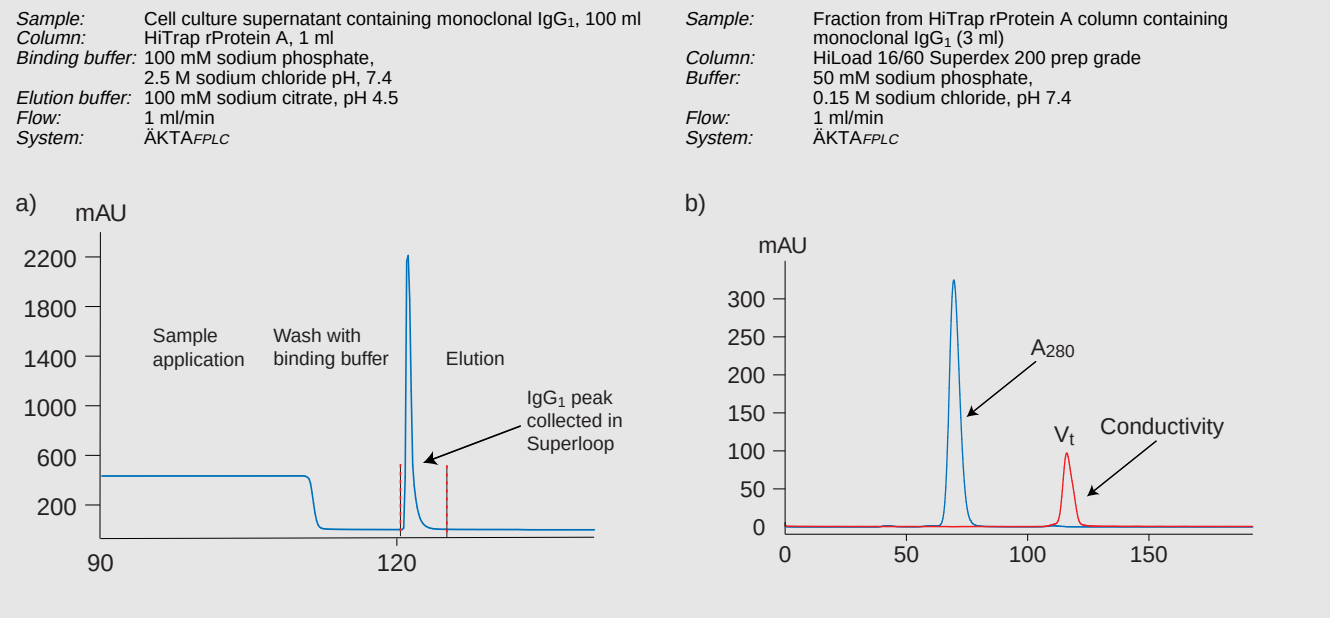
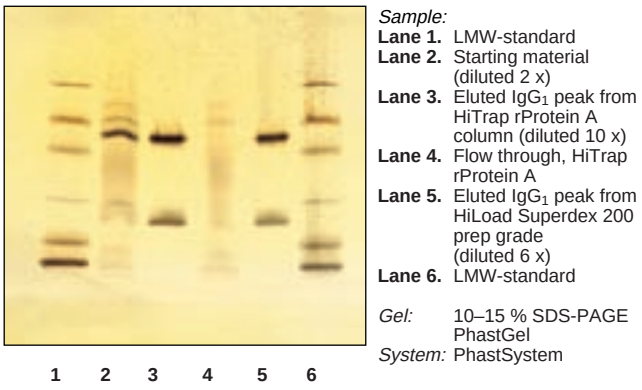


*Fig. 5*a) The optimised capture step on HiTrap rProtein A b) Gel filtration on HiLoad 16/60 Superdex 200 prep grade.



Sample:
Lane 1. LMW-standard
Lane 2. Starting material (diluted 2 x)
Lane 3. Eluted IgG_1 peak from HiTrap rProtein A column (diluted 10 x)
Lane 4. Flow through, HiTrap rProtein A
Lane 5. Eluted IgG_1 peak from HiLoad Superdex 200 prep grade (diluted 6 x)
Lane 6. LMW-standard

Gel: 10–15 % SDS-PAGE PhastGel
System: PhastSystem

Fig. 5c) Purity analysis using SDS-PAGE.

is available in UNICORN. By using the function Method Queue in the software the methods are automatically run after each other.

The results from the automated run are shown in Figure 5.

Analysis

Approximately 1.2 mg MAb was recovered from 50 ml cell culture supernatant. The recovery for both the capture and the polishing step was above 95%. The polishing step removed trace amounts of high material weight material presumably IgG aggregates and/or dimers.

Materials and methods

Materials

All media and columns for chromatography, chromatography systems and equipment and gels for PAGE were from Amersham Biosciences.

Sample

Cell culture supernatant containing mouse monoclonal IgG_1. The concentration was 27 µg/ml (nephelometry). The salt concentration and pH of the sample were adjusted to that of the binding buffer. All samples for the scouting runs had the same volume and protein concentration and were filtered through a 0.45 µm filter.

Affinity chromatography

The antibodies were captured from the cell culture supernatant by HiTrap rProtein A, 1 ml column.

For scouting for optimal salt concentration during binding the column was equilibrated with 100 mM sodium phosphate, 0–3.5 M NaCl pH 7.4. Sample (18 ml cell culture supernatant diluted to 90 ml with buffer) was loaded for each run at a flow rate of 1 ml/min. The salt concentration of the sample was adjusted accordingly. The sample was eluted with 5 ml (5 CV) 100 mM sodium citrate, pH 3.

Sample (46 ml cell culture supernatant diluted to 100 ml with buffer) was loaded in the final method and run at 1 ml/min. After sample application the column was washed with 10 ml (10 CV) binding buffer and finally eluted with 5 ml (5 CV) 100 mM sodium citrate, pH 4.5.

Gel filtration

Fractions collected from the HiTrap rProtein A column were further purified by gel filtration on a HiLoad 16/60 Superdex 200 prep grade column. The column was equilibrated and eluted with 50 mM sodium phosphate, 0.15 M sodium chloride, pH 7.4 and run at a flow rate of 1 ml/min. The IgG peak eluted after approximately 75 ml.

Analysis

Collected fractions were separated and silver stained using PhastSystem™ and 10–15% SDS-PAGE. Separation and staining was performed as described in the protocols supplied with the system.

Chromatography system

ÄKTA*FPLC* with UNICORN control was used for chromatography. The system was equipped with Pump P-920, Monitor UPC-900, Fraction Collector Frac-900, Pump P-50 (for automatic sample injection), two Injection Valves INV-907, four valves PV-908, one valves IV-908, pH flow cell and a pH electrode.

Experimental details are given in the figures.

Trademarks

The following are trademarks owned by Amersham Biosciences or its subsidiaries:
ÄKTA, FPLC, UNICORN, PhastSystem, PhastGel, Superdex, HiTrap, HiLoad.

Ordering Information

Designation	Code No.
ÄKTA*FPLC*	18-1118-67
(254 and 280 nm detection and fraction collector with diversion valve included)	
Computer[1]	
Optional	
UNICORN analysis module	18-1128-56
Zn optics with 214 nm filter	18-1128-21
New functions for alternative system configurations	
On-line measurement of pH conditions	
pH electrode	18-1111-26
pH flow cell including dummy electrode	18-1112-92
Tubing 0.5 mm i.d.(90 mm length required)	18-1113-68
(supplied as accessory with ÄKTA*FPLC* system)	
Increased automation: automatic column, media or buffer scouting	
Buffer selection	
Valve IV-908, including one UniNet cable	18-1108-42
(one valve for pump A, one valve for pump B)	
Column selection	
Valve PV-908, including one UniNet cable	18-1108-41
(2 valves required)	
Automatic multiple sample injection	
Pump P-910, includes pump tubing and UniNet cable	18-1113-86
PV-908, including one UniNet cable	18-1108-41
Tefzel tubing 1.0 mm i.d.	18-1121-16
(supplied as accessory with ÄKTA*FPLC* system)	
Increased security in programmed events – air sensors	
Air-900 control box, includes one UniNet 2 cable, (controls up to 3 air sensors)	18-1121-22
Purge valve (required for buffer inlet position only)	18-1126-33
Air sensor Air-925 (2.5 mm i.d.)	18-1121-24
Air sensor Air-912 (1.2 mm i.d.)	18-1121-23
Optional function valves e.g. for reverse flow, flow diversions	
INV-907, including one UniNet cable (maximum one additional valve of this type)	18-1108-40
IV908, including one UniNet cable (maximum 2 additional valves of this type)	18-1108-42
PV-908, including one UniNet cable (maximum 2 additional valves of this type)	18-1108-41
Control of external equipment	
Communication cable requires 9-pin D-SUB male connector and connector to external equipment (4 digital input/4 digital output signals)	
Pump P-50	19-1992-01

[1] Your local Amersham Biosciences representative will supply details of the fully installed computer to be supplied with the system.

Columns

Designation	Code No.
HiTrap rProtein A	
5 × 1 ml column	17-5079-01
2 × 1 ml column	17-5079-02
HiLoad 16/60	
Superdex 200 prep grade	17-1069-01

Related Product Literature

Designation	Code No.
Application note: Purification and chromatographic characterisation of an integral membrane protein	18-1128-92
Purification on a labile, oxygen-sensitive enzyme for crystallisation and 3D structure determination	18-1128-91
Data File:	
ÄKTAexplorer System Series	18-1124-09
ÄKTApurifier	18-1119-48
Monitor UPC-900	18-1128-40
UNICORN control system	18-1111-20

Please contact your local Amersham Biosciences representativefor further information on ÄKTAdesign high performance chromatography systems.

Amersham Biosciences

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