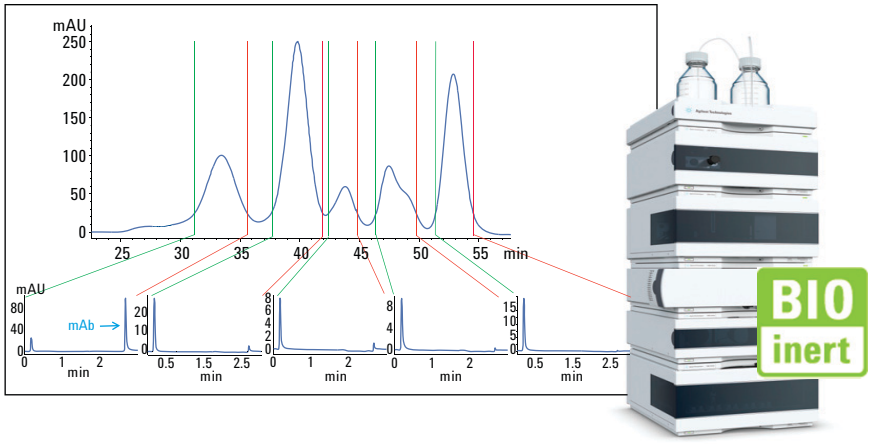
Combining small-scale purification and analysis of monoclonal antibodies on one instrument

Protein purification with high-volume injection using the Agilent 1260 Infinity Bio-inert Quaternary LC System

Application Note

Biopharmaceuticals



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Abstract

This Application Note presents an Application Solution for the small-scale purification of a monoclonal antibody from a cell lysate and the subsequent analysis on a single instrument, the Agilent 1260 Infinity Bio-inert Quaternary LC System. Purification and polishing steps using preparative columns for Protein A purification and size exclusion chromatography were combined with high-volume injection using the Agilent 1260 Infinity Bio-inert Manual Injector. Analysis of the purified mAb was demonstrated using the Agilent Protein A Monolith for quantification and identification. In addition, charge variant analysis was carried out by a four-component ionic strength gradient using ion exchange chromatography, calculated with Agilent Buffer Advisor software.

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Introduction

Preparative and analytical methods for protein purification and analysis require two different instrument setups. Protein preparation requires large injection volumes and high flow rates, whereas analytical methods require small injection volumes and lower flow rates. Traditionally, they are done on two instruments specifically designed for these purposes.

With the 1260 Infinity Bio-inert Quaternary LC, both tasks can be done on a single instrument. This Application Note shows a two-step purification procedure for monoclonal antibodies (mAb) out of cell lysate using Protein A affinity chromatography followed by a size exclusion polishing step. Subsequently, the purified mAb fraction was, on an analytical scale, identified and quantified with Protein A affinity chromatography and the charge variants were characterized with ion exchange chromatography (IEX).

E.coli cell lysate was used as a surrogate model system for a hybridoma cell culture supernatant for the small-scale purification of a monoclonal antibody. Only in rare cases is a single step purification procedure sufficient to achieve the desired purity. In general, at least three purification steps are necessary for high protein purity: capture, intermediate, and polishing. Therefore, in this Application Note a two-step purification method was used, consisting of a capture and a polishing step. In the first step, the mAb is captured by affinity chromatography using Protein A. Protein A is a surface protein originally isolated from the bacterium *Staphylococcus aureus* with a size of 56 kDa. Protein A binds among other immunoglobulin (Ig) G variants with a high affinity to human IgG1 and is, therefore, the most common and accepted technique for antibody purification[1]. The polishing step using size exclusion chromatography removes any remaining trace contaminants, resulting from unspecific binding to column packing material, for example. For higher levels of purity, additional purification steps can be added prior to the SEC polishing such as IEX[2]. Various chromatography techniques using different selectivities form powerful combinations for biomolecule purification.

The monoclonal antibody used in this workflow was the anti-c-Myc antibody, displayed in Figure 1 in an IEX ionic strength gradient resolving small acidic and basic charge variants.

The analysis of the purified mAb after the SEC polishing step is an essential part of the workflow to determine purity and quantity. Especially for pharmaceutically-used mAbs, it is extremely important to thoroughly characterize the biomolecules to ensure efficacy and safety of the drug. One part of the characterization procedure, besides peptide mapping and aggregate analysis, is the determination of charge variants of the mAb, usually carried out by ion exchange chromatography. In this Application Note, the analysis of the purified mAb includes, besides identification using Protein A chromatography, also quantification. The characterization of the mAb in terms of charge variants was carried out using weak cation exchange chromatography in a 4-component ionic strength gradient – calculated with Agilent Buffer Advisor software.

To enable the combination of preparative and analytical techniques for biomolecules in one instrument, high volume injection is required for the preparative part. This can be achieved using the 1260 Infinity Bio-inert Manual Injector and bio-inert PEEK loops, which are available up to 20 mL.

This Application Note shows the purification of the monoclonal antibody anti-c-Myc out of 5 mL *E.coli* cell lysate with subsequent polishing and analysis on the 1260 Infinity Bio-inert Quaternary LC.

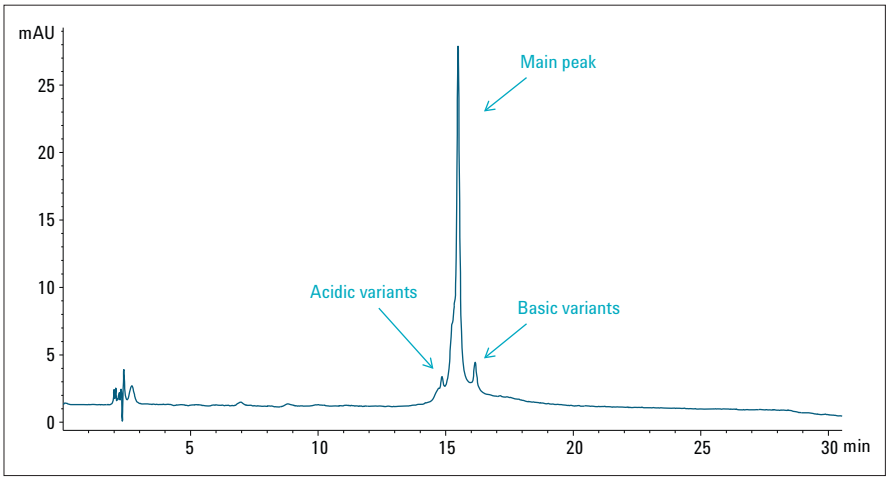


Figure 1
IEX chromatogram of anti-c-Myc monoclonal antibody.

Experimental

The Agilent 1260 Infinity Bio-inert Quaternary LC System consisted of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A)
- Agilent 1260 Infinity High performance Bio-inert Autosampler (G5667A)
- Agilent 1290 Infinity Thermostat (G1330B) for sample cooling
- Agilent 1290 Infinity Thermostat (G1330B) for fraction cooling
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C) with bio-inert solvent heat exchanger
- Agilent 1260 Infinity Diode-Array Detector VL (G1315D with bio-inert standard flow cell, 10 mm)
- Agilent 1260 Infinity Bio-inert Analytical-scale Fraction Collector (G5664A)
- Agilent 1260 Infinity Bio-inert Manual Injector (G5628A)
- Connecting capillaries: 0.5 mm (id) PEEK

Columns

- HiTrap Protein A HP, 1 mL (GE Healthcare, Little Chalfont, United Kingdom)
- Superdex 200, 10/300 GL, 13-µm, 10 × 300 mm (GE Healthcare, Little Chalfont, United Kingdom)
- Agilent Protein A Monolith
- Agilent Bio MAb, 4.6 × 250 mm, 5-µm, PEEK

Software

- Agilent OpenLAB CDS ChemStation Edition for LC & LC MS Systems, Rev. C.01.04 [35]
- Agilent Buffer Advisor, Rev. A.01.01

Sample

- Agilent Anti-c-Myc Monoclonal Antibody, Clone 9E10 spiked into 5 mL *E.coli* cell lysate

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-µm membrane point-of-use cartridge (Millipak). PBS (phosphate buffered saline) was purchased from PAA (GE Healthcare, Little Chalfont, United Kingdom). MES (2-(N-morpholino) ethanesulfonic acid monohydrate) and MES-Na (2(N-morpholino)ethanesulfonic acid sodium salt) were purchased from Merck, Darmstadt, Germany. NaCl was purchased from VWR, Radnor, PA, USA. *E.coli* cells and the CelLytic B Plus Kit were purchased from Sigma-Aldrich, St. Louis, MO, USA. Amicon Ultra Centrifugal Filters 30K were purchased from Millipore, Billerica, MA, USA.

Solvents and samples

	Protein A purification	SEC polishing	Protein A analysis	IEX – 20 mM MES (Buffer Advisor calculation)
Buffer A	PBS	PBS	PBS	H_2O_{dd}
Buffer B	Glycine-HCl, 100 mM, pH 3		Glycine-HCl 100 mM, pH 3	NaCl, 1 M
Buffer C				MES, 60 mM
Buffer D				MES-Na, 35 mM

Table 1
Solvents.

Chromatographic conditions

	Preparative	
	Protein A purification	**SEC polishing**
Flow rate:	0.5 mL/min	0.5 mL/min
Column:	HiTrap Protein A HP, 1 mL (GE Healthcare)	Superdex 200, 10/300 GL, 13 µm, 10 × 300 mm (GE Healthcare)
Gradient:	0.00 minutes 100% A 40.00 minutes 100% A 41.00 minutes 100% B 65.00 minutes 100% B Stop time: 65 minutes	Isocratic - 100% A Stop time: 80 minutes
Injection volume:	5 mL (manual injection using 5 mL bio-inert PEEK sample loop)	500 µL (manual injection using 500 µL bio-inert PEEK sample loop)
Temperature TCC:	RT	RT
Thermostat fraction collector:	6 °C	6 °C
DAD:	280 nm/4 nm	280 nm/4 nm
Peak width:	Ref.: OFF	Ref.: OFF
	> 0.05 minutes (1.0 second response time) (5 Hz)	> 0.05 minutes (1.0 second response time) (5 Hz)
Fraction collection		
Peak detector mode:	Threshold only → 10 mAU	Threshold only → 20 mAU
Fraction trigger mode:	Peak-based with a maximum peak duration of 3 minutes	Peak-based with a maximum peak duration of 10 minutes
Fraction trigger timetable:	0 minutes → off 40 minutes → on	–

Due to the separation technique of the SEC polishing step, the fractionated samples were diluted to a large extent. Before analytical chromatography could be applied to the sample, a concentration step was carried out by ultrafiltration using Amicon Ultra Centrifugal Filters 30K.

The IEX 4-component ionic strength gradient from 0 to 200 mM NaCl in 25 minutes was calculated with the Agilent Buffer Advisor software for 20 mM MES buffer concentration at pH 6.4. The best pH value for separation was estimated by pH scouting using Buffer Advisor (data not shown).

	Analytical	
	Protein A monolith	**IEX**
Flow rate:	0.75 mL/min	
Column:	Agilent Protein A monolith	Bio MAb column, 4.6 × 250 mm, 5µm
Gradient:	0.50 minutes 100.0% A 1.50 minutes 100.0% B 3.00 minutes 100.0% A Stop time: 3 minutes Post time: 5 minutes	0.00 minutes A: 51.4% B: 0.0 % C: 11.9% D: 36.7% 25.00 minutes A: 30.7% B: 20.0% C: 11.1% D: 38.2% 30.00 minutes A: 0.9% B: 50.0% C: 11.3% D: 37.8% 35.00 minutes A:0.9% B: 50.0% C: 11.3% D: 37.8% 36.00 minutes A: 51.4% B: 0.0% C: 11.9% D: 36.7% Stop time: 36 minutes Post time: 20 minutes
Injection volume:	0.156 to 40 µL	20 µL
Temperature TCC:	RT	
Thermostat Autosampler and Fraction collector:	6 °C	
DAD:	280 nm/4 nm	
Peak width:	Ref.: OFF	
	> 0.05 minutes (1.0 second response time) (5 Hz)	

Results and discussion

A 5 mL *E.coli* lysate solution was spiked with the monoclonal antibody anti-c-Myc and injected into the 1260 Infinity Bio-inert Quaternary LC System using the 1260 Infinity Bio-inert Manual Injector with a 5-mL bio-inert PEEK loop.

Using a preparative affinity column coated with Protein A, the mAb could be purified out of the *E.coli* lysate, see Figure 2. The mAb was captured and successfully separated from the constituent parts of the *E.coli* cell lysate with an enormous protein content of over 100 mg/mL. The system could be operated at extremely low backpressure due to the low backpressure of the preparative column, the manual injector and PEEK capillaries with 0.5-mm id. The system backpressure of the complete run was below 5 bar with less than 1-bar backpressure over the column.

The second step of the purification procedure using SEC revealed several non-specifically bound proteins after the Protein A capture step, see Figure 3A. Again, the system could be operated at very low backpressure not exceeding 10 bar. Five peaks were fractionated and the fractions concentrated by ultrafiltration for further analysis with Protein A affinity chromatography and IEX.

Figure 3B displays identification using Protein A affinity chromatography as the first part of the mAb analysis. The first peak in the SEC chromatogram shows the presence of mAb, because the corresponding Protein A analysis showed significant retention. Conversley, none of the other fractions showed representative retention, demonstrating that their content is not captured by Protein A.

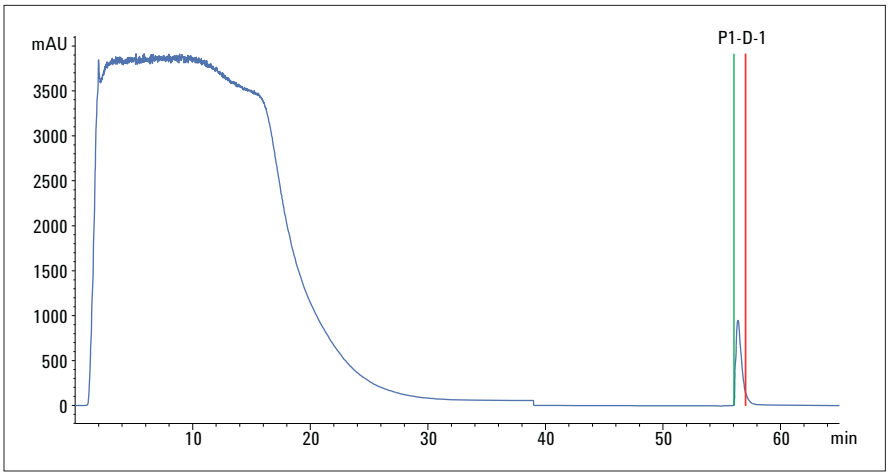


Figure 2
MAb purification using Protein A affinity chromatography.

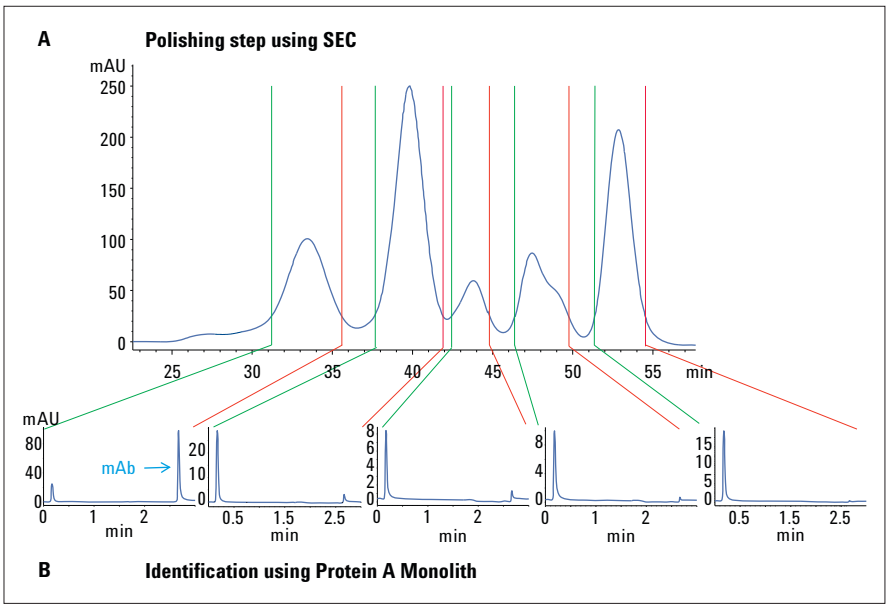
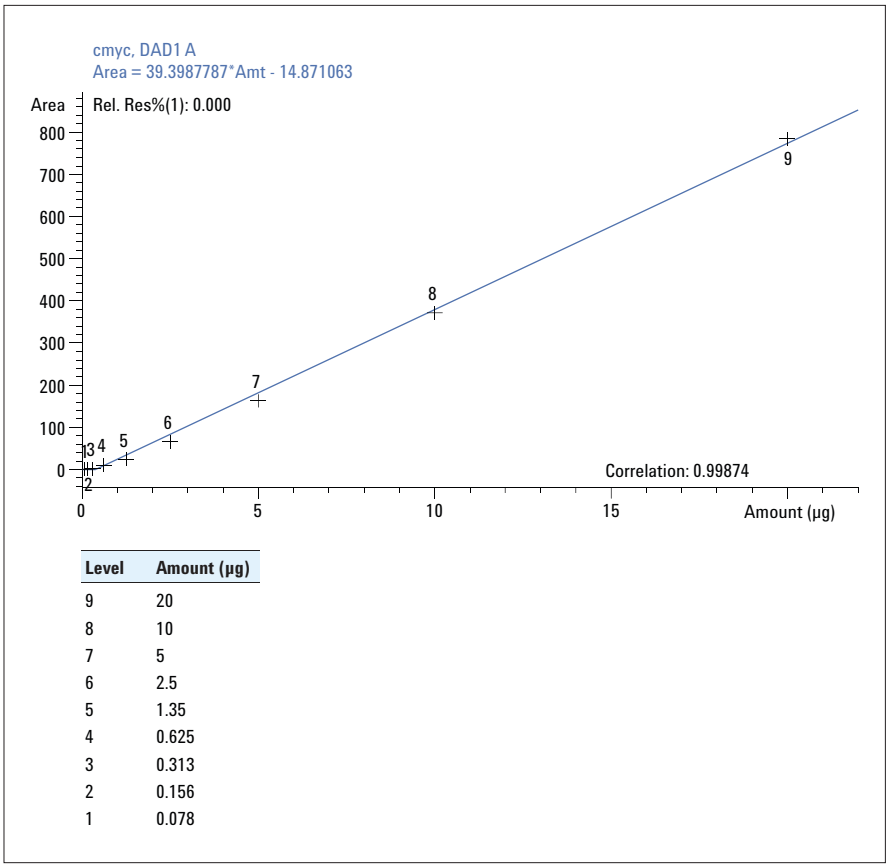


Figure 3
A) Polishing step using SEC and B) identification of the fractionated mAb using Protein A affinity chromatography.

Several pitfalls, regarding manual injection and fraction collection, can be easily avoided by considering following steps:

- Connect the pump to the fraction collector in the *Configuration* options under *Linked pump*. This important setting avoids overfilling of the fractionation vials or wells due to the correct communication of the flow rate.
- Start the run over *Single sample* to ensure that the fraction collection starts in the correct location as specified in the *Sample info* options.
- Insert a vial position into the *Sample parameters* within the S*ample info* option even though the manual injector is used. Otherwise, the system is going to perform a blank run.

In addition to identification, the purified mAb was quantified with Protein A affinity chromatography using a calibration curve, measured with pure anti-c-Myc. The calibration curve showed a good correlation factor of 0.99874 from 78 ng to 20 µg total protein on the column, see Figure 4. The quantification of the purified anti-c-Myc resulted in a concentration of 0.94 mg/mL.



Level	Amount (µg)
9	20
8	10
7	5
6	2.5
5	1.35
4	0.625
3	0.313
2	0.156
1	0.078

Figure 4
Correlation curve of anti-c-Myc with Protein A affinity chromatography.

As the final step, the purified mAb was analyzed for charge variants using a 4-component ionic strength gradient at pH 6.4, calculated with Agilent Buffer Advisor software. The IEX chromatogram reveals some small acidic and basic charge variants of the anti-c-Myc mAb, see Figure 5.

The chromatogram looks nearly identical compared to the pure anti-c-Myc monoclonal antibody before it was spiked into the *E.coli* lysate (Figure 1). Hence, the purification was considered successful.

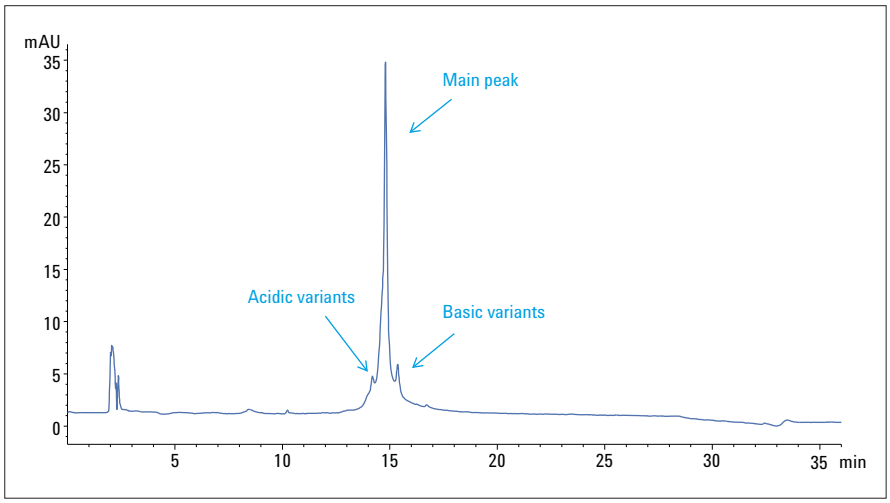


Figure 5
Charge variant analysis of the purified anti-c-Myc monoclonal antibody.

Conclusion

This Application Note shows the combination of small-scale protein preparation and subsequent analysis on a single instrument, the Agilent 1260 Infinity Bio-inert Quaternary LC. A two-step purification procedure using preparative columns was applied to purify the monoclonal antibody anti-c-Myc from *E.coli* cell lysate. The first step using Protein A affinity chromatography captured the anti-c-Myc and separated the mAb from other constituent parts of the *E.coli* cell lysate. The SEC polishing step revealed several unspecific bound proteins after the Protein A affinity capture, which were fractionated and successfully separated from the mAb as the target molecule. Both purification steps required high volume injection using the 1260 Infinity Bio-inert Manual Injector. In addition, both purification steps could be operated at extremely low backpressure below 10 or even below 5 bar total system backpressure.

After fractionation and concentration of the sample, the mAb was analyzed regarding identity, quantity, and charge variants. A calibration curve of anti-c-Myc over approximately four orders of magnitude revealed good correlation with a correlation factor of 0.99874 for the quantification using the Protein A Monolith. In addition, charge variant analysis was carried out by a four-component ionic strength gradient, calculated with Buffer Advisor software.

In summary, the Agilent 1260 Infinity Bio-inert Quaternary LC enables small-scale preparative and analytical workflows on a single instrument.

References

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