HPLC solution for the removal of high abundance proteins in human serum

Application Note

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Abstract

Agilent Technologies recently introduced the Agilent Multiple Affinity Removal System for depletion of six high-abundance proteins from human serum. This system includes an affinity column with immobilized antibodies to remove albumin, IgG, IgA, haptoglobin, transferrin, and anti-trypsin, as well as optimized buffers for affinity capture and subsequent elution of these high-abundance proteins. In this Application Note we present a complete Agilent 1100 HPLC solution and corresponding methods suited to work with the Multiple Affinity Removal System. This solution allows the investigator to perform multiple runs in an automated fashion in combination with fraction collection. Pooling of fractions, and cooling of valuable samples and fractions can be conducted according to the investigator's needs.

Agilent Technologies

Introduction

Dynamic range is a very critical issue in proteomics. In many cases low abundance proteins are most promising drug targets or provide the key for understanding pathogenesis in a disease. However, current technologies such as 2D gel electrophoresis and 2D HPLC in combination with MS and MS/MS are limited in coping with a dynamic range greater than 10^4 and 10^5, respectively. In serum and other body fluids concentration differences of expressed individual proteins can differ by a factor of 10^9 to 10^{12}. Therefore, efficient prefractionation techniques are an important prerequisite to detect physiologically interesting lower abundance proteins.

In human serum, the six most abundant proteins sum up to 85 % -90 % of the total protein mass. The novel Agilent Multiple Affinity Removal System specifically binds these proteins and removes them in a single step procedure[1]. Low abundance proteins are collected as flow-through and can be further downstream-processed while bound high abundance proteins are finally eluted using a second Buffer (Buffer B). The column itself can be regenerated and reused for at least 200 injections. This provides the investigator with the possibility to process either larger amounts of an individual serum sample or to analyze multiple samples with a single column. We recommend a complete hardware and software LC solution that allows multiple automated runs and fulfills typical user requirements such as monitoring, precise fraction collection and cooling samples and recovered fractions.

Experimental

Equipment

Agilent 1100 Series HPLC system:
The following LC components were used: Quaternary pump with seal wash option, degasser, solvent cabinet, preparative autosampler (900 µL loop) with thermostat, diode-array detector with standard flow cell, analytical scale fraction collector with 0.25-mm capillaries, semi-prep needle and thermostat and thermostatted
column compartment with micro 2-position/6-port valve (figure 1). Detailed ordering information is provided on page 6.

Software:
ChemStation A 10.01

Agilent Multiple Affinity Removal System:
Columns: 4.6 x 50 mm for 15-20-µlL serum capacity or 4.6 x 100 mm for 30-40 µL serum capacity.
Reagents: Buffer A for loading, washing, equilibration; Buffer B for eluting high abundance proteins, Human Serum Albumin (HSA) standard (20mg/mL in Buffer A), spin filters for sample pre-cleanup, spin concentrators with 5KDa MWCO exclusion size.

Accessories: 4-well-plate tray for fraction collection (Agilent Technologies), 24 well-plates (10 mL; Whatman, Maidstone, UK) for pooling or 31 mm 96 deep well-plates (Agilent Technologies) for collection of single fractions. A Human Serum sample was purchased from Sigma-Aldrich (Taufkirchen, Germany).

Chromatographic method

Chromatography was performed according to the instructions supplied with the columns[2]. Pump timetables for the 50-mm and the 100-mm column are shown in figure 2. In brief, sample (15 and 35 µL

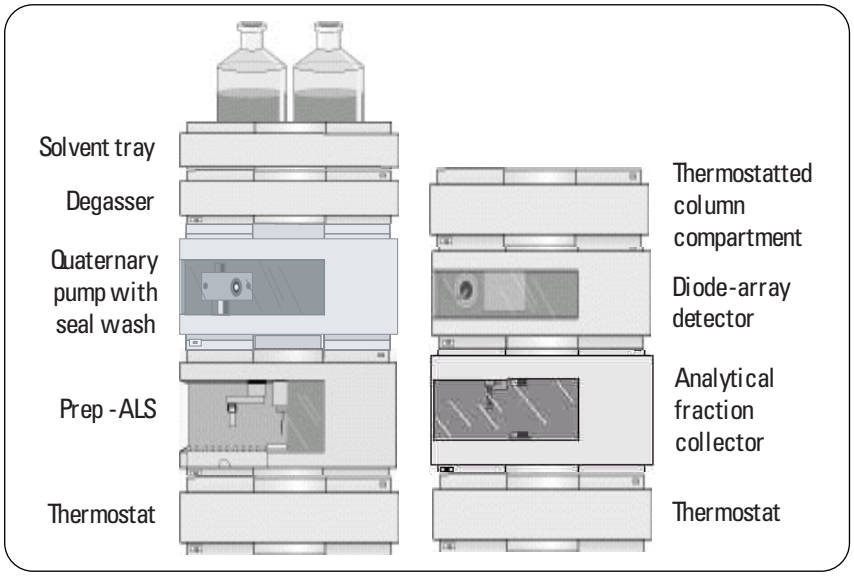

Figure 1
Recommended Agilent 1100 Series HPLC system to be used with the Agilent Multiple Affinity Removal System

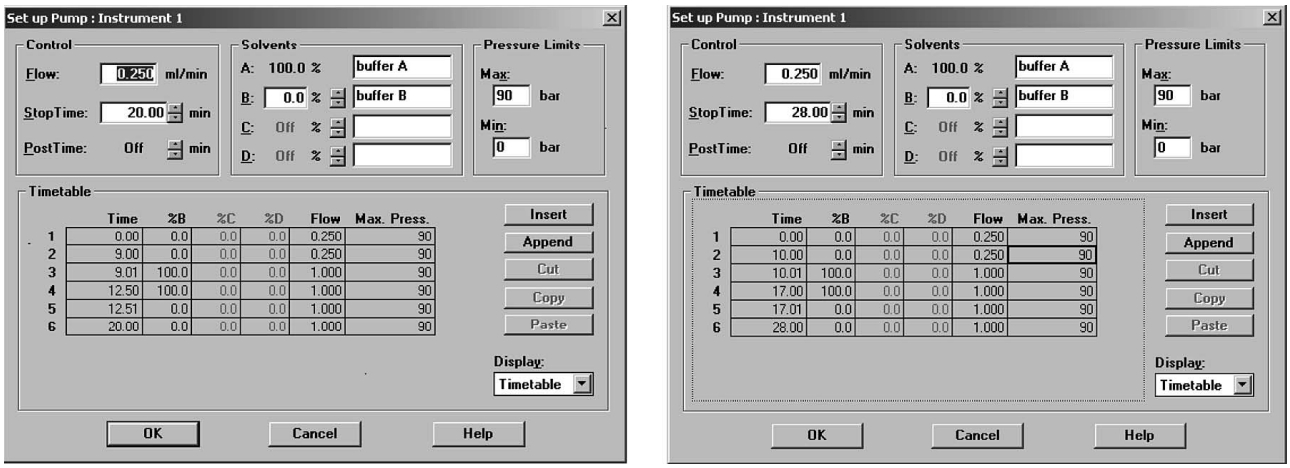

Figure 2
Chromatographic time table used for the 4.6 x 50 mm (left) and 4.6 x 100 mm affinity column (right)

of serum diluted with 4 volumes Buffer A, for the 50-mm and the 100-mm column, respectively) was filtered and loaded onto the column. After 9 and 10 minutes, respectively, elution of high abundance proteins was started with Buffer B at a flowrate of 1 mL/min. Final re-equilibration times with Buffer A are shown in figure 2. In order to ensure fast buffer delivery on the column after switching buffers, an injection program was set up to bypass the 900 µL sample loop following column loading (figure 3). This greatly reduces delay volumes and allows faster sample analysis times.

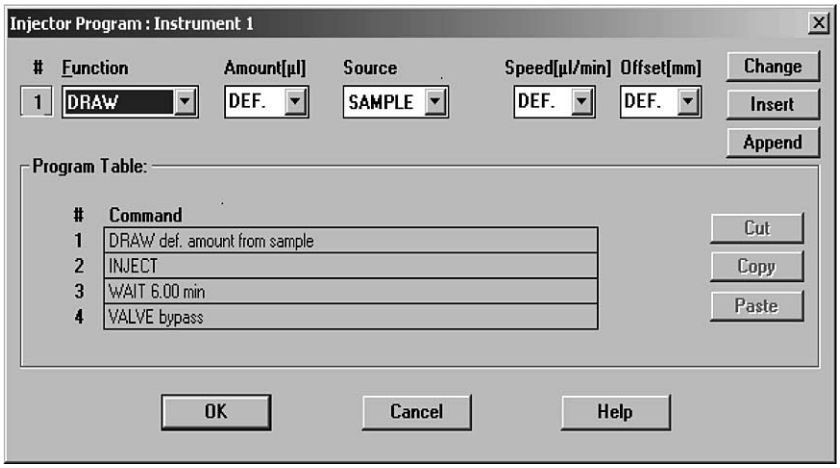

Figure 3
Injection program used for both affinity columns allowing fast solvent change from Buffer A to B and vice versa

Fraction collection

Fraction collection was time-controlled as shown in the timetable of the fraction collector (figure 4). Two fractions were recovered – the first contained low-abundance serum proteins from the column flow-through, the second contained eluted high abundance proteins. Fraction collection was performed for single runs as well as for repeated sequential runs where corresponding fractions were pooled in 8-mL 24 well-plates. Samples and fractions were cooled to 4 °C using thermo-stats connected to the autosampler and the fraction collector.

Sample processing

Serum samples were diluted with 4 volumes of Buffer A and filtered prior to injection to remove particles using 0.22-µm spin-filters. Recovered fractions after affinity removal were concentrated to the desired volume and buffer exchange to 10 mM Tris-HCl pH 7.4 was performed using spin concentrators with an exclusion mass of 5 kDa.

Analysis of proteins

For quality control, fractions were re-analyzed for protein content using the Agilent 2100 bioanalyzer with the Protein 200 Plus assay.

Results and discussion

HPLC system description

Various series of experiments were carried out in order to best provide the investigator with a recommendation on how to optimally exploit the technology of the Agilent Multiple Affinity Removal System within sophisticated HPLC instrumentation. Analysis of proteomic serum samples often requires either high throughput of a number of different samples or processing of larger volumes of a single serum sample to bring low abundant proteins of interest into detectable levels. Therefore, reproducibility in solvent delivery, automation, cooling of degradable samples, and precise fraction collection are highly important instrument features that guarantee desired results in this initial step of serum proteome analyses.

The Agilent 1100 Series system suggested here consists of a quaternary pump with seal wash option permitting pump seals to be washed during analyses with buffers containing high salt concentration. In addition, fast cleaning of the system with water after finishing sample processing is possible (and strongly recommended) using the additional solvent delivery channels of the pump.

With the automated preparative autosampler connected to a thermostat, samples up to 900-µL from a variety of preconfigured sample vials can be analyzed. A user defined ChemStation injector program (figure 3) that bypasses the injection loop provides the option for low buffer-exchange times. The thermostatted column compartment gives investigators the opportunity to adjust the temperature during separation for optimum performance. Fraction delay volumes can be calibrated using the Agilent 1100 Series analytical fraction collector (with the fraction collector's delay sensor) in combination with the diode-array detector. This ensures correct deposition of sharply cut fractions. The 4-well-plate tray of the fraction collector provides room for recovery of 96 fractions of 8 to 10 mL (in 24 well-plates) or in any other preconfigured or user-defined vial or well-plate. The cooling option for samples and fractions minimizes degradation. Finally, ChemStation rev. 10.1 offers flexibility in fraction collection, including pooling of fractions from repeated runs, recovery on the track, peak-based or time-based fraction collection and automation of sample processing.

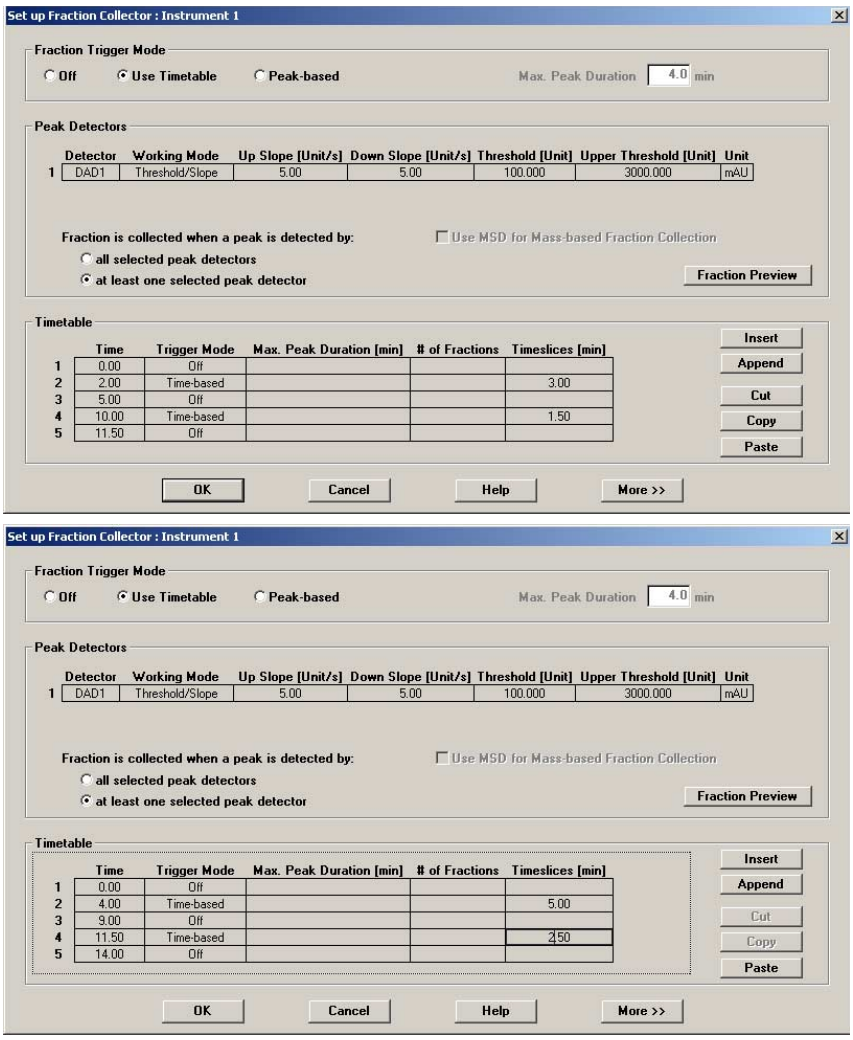

Figure 4
Fraction collection setup, time-based triggered for the 50 mm (up) and the 100 mm (down) affinity column

Agilent 1100 HPLC Series systems performance with the Multiple Affinity Removal System

Repeated and single injections of human serum were done with the two affinity column dimensions (4.6 x 50 mm and 4.6 x 100 mm) using commercially available human serum samples. Samples were processed sequentially using the ChemStation Sequence Table features. Corresponding fractions of either high or low-abundance-proteins were pooled automatically by the analytical fraction collector. Overlay chromatograms demonstrate excellent reproducibility of repeated runs with 15 µL serum for the 50 mm affinity column or 40 µL serum for the 100-mm column (figure 5). No carryover of sample was observed in blank runs following sequences of multiple serum injections. High abundance proteins were removed from the sample almost quantitatively as demonstrated by the UV absorbance track of injected Human Serum Albumin HSA, where with both columns the albumin (up to 1 and 2 mg) is eluted completely in the high-abundance fraction (figure 6) as well as by fraction analysis with the Agilent 2100 bioanalyzer (figure 7). The lanes shown for the low abundance fractions clearly show a dramatic enrichment of these protein species while high-abundance proteins such as albumin are almost absent.

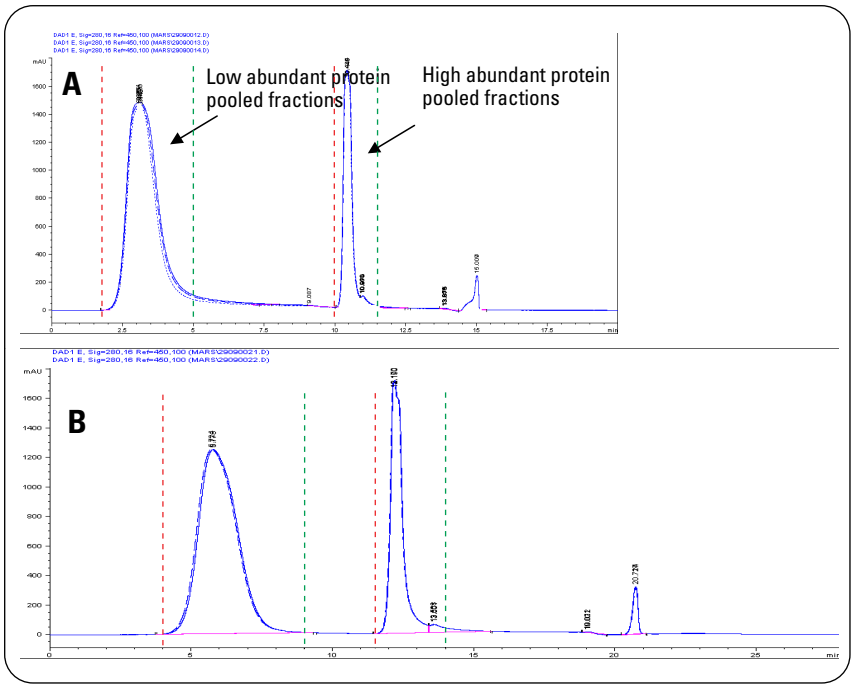

Figure 5
Repeated injection of serum with pooled fraction collection using the Agilent 1100 Series HPLC system. A: 50 mm column, panel B: 100 mm column.

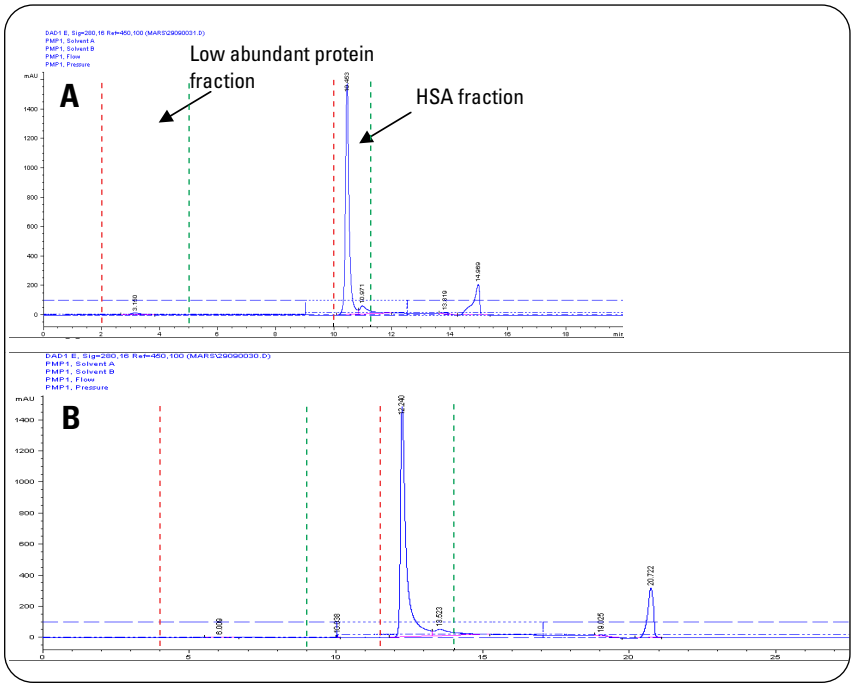

Figure 6
Removal of Human Serum Albumin (HSA) using the Agilent Multiple Affinity Removal System and the recommended Agilent 1100 Series HPLC system.
A: 50-mm column, 0.3 mg HSA injected B: 100-mm column, 0.8 mg injected. Vertical lines indicate start (red) and end (green) of the collected fractions.

Conclusion

The Multiple Affinity Removal System is excellently suited to work in a highly automated Agilent 1100 Series HPLC system. The recommended system provides cooling

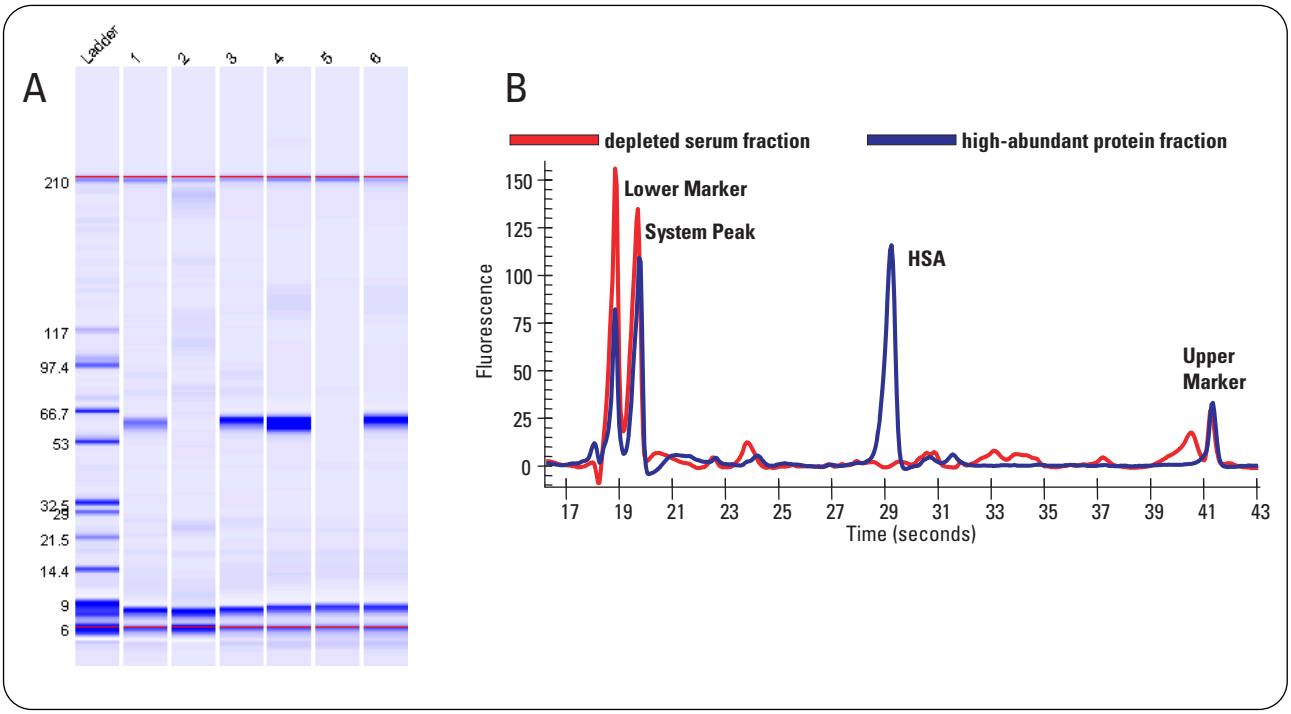

Figure 7
A: Gel-like image of serum samples analyzed with the Agilent 2100 Bioanalyzer (Protein 200 Plus Assay). Serum and HSA samples are shown before and after depletion using the Multiple Affinity Removal System on the recommended Agilent 1100 series HPLC. Upper and lower marker bands are seen in all lanes. Lanes: (1) Serum prior to chromatography, (2) depleted serum fraction, (3) high-abundant protein fraction, (4) HSA solution prior to chromatography, (5) depleted fraction from HSA sample, (6) HSA fraction after chromatography.
B: Electropherogram comparing high-abundant protein fraction (blue) and depleted serum fraction (red).

for both fractions and starting samples and is able to work reliably and reproducibly with buffers containing high salt concentrations. Up to 7 or even 10 low-abundance fractions can be pooled in a single well (using 24 well-plates) with the 100-mm and the 50-mm columns, respectively. This finally allows automated pooling of the low abundance proteins from initially 280 µL and 200 µL of serum sample depending on the choice of column dimension.Thus, automated and unattended pooling of low abundance proteins in a single well from up to 280 µL of human serum sample using the Agilent 1100 HPLC fraction collection system is now feasible.

Ordering Information	
Multiple Affinity Removal System	
5185-5984	4.6 x 50 mm column
5185-5985	4.6 x 100mm column
5185-5986	Starter kit (buffers, concentrators, filters)
5185-5989	HSA standard
Hardware	
G1354 A	Quaternary pump (with seal wash option 01018-68722), degasser, solvent cabinet
G2261A	Preparative autosampler (900 µL loop) + thermostat
G1315 B	Diode-array detector + flow cell #012
G1364 C	Analytical scale fraction collector + G1330 B thermostat, semi prep needle, 0.25-mm tubing
G1316 A	Thermostatted column compartment
Protein analysis	
G2940CA	2100 bioanalyzer desktop system
G2947CA	Electrophoresisset
Software	ChemStation rev 10.01
Acessories	
G1364-84501	4-well-plate-tray (alternatively: 2-well-plate, 10-port funnel tray plates (G1364-84502)
5042-6454	31-mm deep 96-well-plate (Agilent, Nunc) for collection of fractions from single runs
From Whatman	24 well-plate (10 mL, with lid) for pooling of fractions from multiple runs (7701-5110)

References

1.
"Agilent Multiple Affinity Removal System for the Depletion of High-Abundance Proteins from Human Serum.", Zhang, K; Zolotarjova, N; Nicol, G; et al, *Agilent Application Note 5988-9813EN*, **2003.**

2.
"Agilent Multiple Affinity Removal System for the Depletion of High-Abundance Proteins from Human Proteomic Samples.", *Instruction Manual provided with the affinity capture column*, **2003.**

3.
"Use of Lab-on-a-Chip Technology for Protein Sizing and Quantitation.", M. Kuschel, T. Neumann, P. Barthmaier and M. Kratzmaier, *J. Biomolecular Techniques; 13(3), 172-178*, **2002.**

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