Rapid process development for purification of a MAb using ÄKTA avant 25

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Rapid process development for purification of a MAb using ÄKTA™ avant 25

ÄKTA avant 25 system controlled by UNICORN™ 6 software was used to develop a two-step chromatography process for purification of a monoclonal antibody (MAb). MabSelect SuRe™, a protein A-based chromatography medium (resin) was used for the initial capture step while the multimodal anion exchanger Capto™ adhere was used for reduction of impurities in a second, polishing step. An experimental design was applied to the polishing step to screen loading conditions. Sample pH, conductivity, and load were varied. Using 1 ml prepacked HiTrap™ columns, the design was run in less than 24 h and a reduced design was performed to establish the robustness of the process conditions. Using the Design of Experiments (DoE) functionality incorporated in UNICORN 6 together with HiScreen™ prepacked columns, optimization of the overall process was achieved in approximately one week. Despite the challenging nature of the feed studied, high yield and purity of the target MAb was achieved.

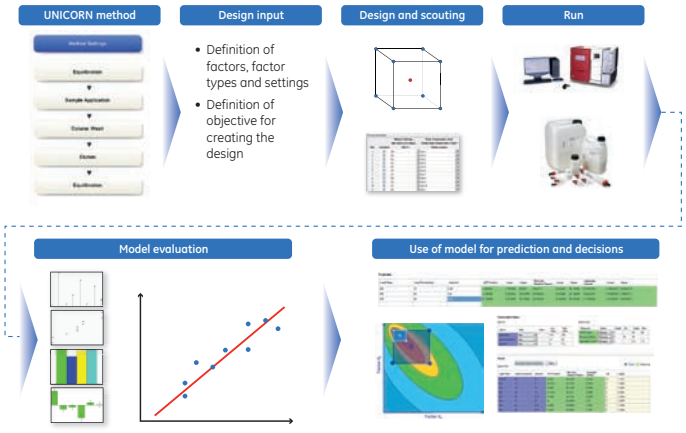


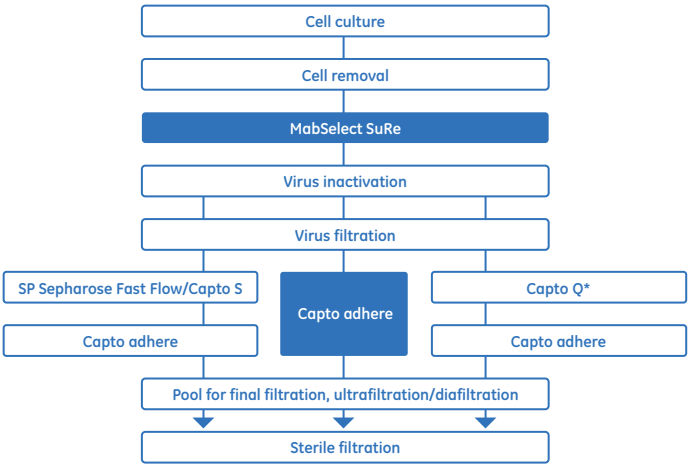
Fig 1. UNICORN 6 software with built-in DoE functionality is designed for easy and fast process development. In DoE, multiple factors are varied simultaneously and the resulting data is used to generate a statistical model. The model is validated and used to produce maps of the system to support decision making.

Introduction

Time and flexibility are essential in process development of purification processes for biopharmaceutical compounds. ÄKTA avant 25 is a chromatography system designed for fully automated process development using rigid chromatography media. Productivity is enhanced by the Design of Experiments (DoE) software functionality incorporated in UNICORN 6. DoE (Fig 1) facilitates easy and fast process development, is a powerful tool for optimizing chromatography conditions, and increases throughput in the process development lab.

In the purification of monoclonal antibodies (MAb), protein A is the chromatography medium of choice for capture, due to its high selectivity giving excellent purity and ease of use at large and small scale. Therefore, protein A-based media are the basis for the platform approach to MAb purification.

Subsequent downstream processing can be performed according to a variety of chromatography techniques and combinations of techniques (especially ion exchange and hydrophobic interaction chromatography). A novel approach to MAb purification involves a two-step process (Fig 2) whereby a multimodal chromatography medium, capable of both anion exchange as well as other types of interaction, enables the selective removal of antibody dimers and aggregates (D/A), and removal of host cell protein (HCP), DNA, and viruses at the same time (1, 2). In order to take full advantage of the beneficial properties of a multimodal chromatography medium, a thorough purification process optimization is required.



* WO 2004/076485

Fig 2. A classical three-step antibody purification strategy employing MabSelect SuRe, SP Sepharose™ Fast Flow, and Capto adhere (left arrow); two-step strategy based on MabSelect SuRe and Capto adhere (center arrow); and alternative three-step strategy using Capto media for polishing (right arrow).

The following steps (summarized in Table 1) were taken in the development of a method for purification of a MAb using ÄKTA avant 25 controlled by UNICORN 6: (1) an affinity chromatography step was initiated by a pH-elution experiment to determine a suitable pH range for elution of the MAb; (2) analysis was performed to determine dynamic binding capacity; (3) purification of the MAb from feed was performed; (4) the purification method was scaled up to prepare material for the polishing step; (5) purified antibody pools were used for study of loading conditions for the polishing step; (6) parameter screening using DoE was performed; (7) a robustness study using DoE was executed.

Materials and methods

Columns, equipment, and purification of MAb

For this MAb purification, UNICORN 6 software was used to control ÄKTA avant 25 system configured with two sample inlet valves, which automates sample loading and wash. Runs were performed using the pressure/flow regulation feature of ÄKTA avant 25, which ensures that no excessive pressure builds up during sample loading. Essentially the same method was used for all affinity chromatography runs using MabSelect SuRe and for all ion exchange runs using Capto adhere. Prepacked columns of MabSelect SuRe and Capto adhere in HiTrap (1 ml) and HiScreen (4.7 ml) formats were used for the various steps of the experiment (Table 1). An XK 50/20 column packed with MabSelect SuRe was used to purify material for optimization of the polishing step (step 4, Table 1).

Design of Experiments for screening of loading conditions on Capto adhere

Conductivity, pH, and sample load are known to be important parameters for sample loading on Capto adhere (1). An experimental setup should be designed to test many different conditions (factors) simultaneously. This is preferably performed using Design of Experiments (DoE), which employs statistics to identify and define the factors having the greatest impact on the process/product. A carefully selected set of experiments is devised in which all relevant factors are varied and evaluated simultaneously to maximize the information gained. In method optimization, the use of DoE greatly increases the probability that the real purification optimum is established.

In this study, the full factorial Design of Experiments (DoE) included in UNICORN 6 was used for screening of sample loading conditions on Capto adhere (see step 6, Table 1). This full factorial design consisted of three parameters (sample load, conductivity, and pH), that is $2^3 = 8$ experiments and 3 center points giving a total of 11 experiments (Table 2). The center points corresponded to a sample load of 192 mg/ml medium, conductivity of 20 mS/cm, and pH 6.75. The starting material concentration varied between 10.8 and 11.7 mg MAb/ml, HCP at an average of 45 ppm, protein A at 2 ppm, and D/A at 1.9%.

Table 1. Summary of the different columns and conditions used

Step no.	Description and purpose	Column	Buffer
1	Determination of elution pH on MabSelect SuRe	HiScreen MabSelect SuRe	20 mM citrate, pH 6.0 to 3.0
2	Determination of dynamic binding capacity with MabSelect SuRe	HiTrap MabSelect SuRe	PBS, pH 7.4 and citrate, pH 3.5
3	Purification of MAb on MabSelect SuRe	HiScreen MabSelect SuRe	PBS, pH 7.4 and citrate, pH 3.5
4	Preparation (scale-up) of material for Capto adhere polishing step	MabSelect SuRe packed in XK 50/20 column (bed height = 8.4 cm, V_c = 165 ml)	PBS, pH 7.4 and citrate, pH 3.5
5	Determination of loading conditions on Capto adhere	HiScreen Capto adhere	20 mM sodium phosphate, 20 mM citrate, pH 7.8 to 4.0
6	Design of Experiments (DoE) for screening of loading conditions on Capto adhere	HiTrap Capto adhere	20 mM sodium phosphate, 20 mM citrate, pH 7.5, 6.75, and 6.0
7	Robustness study on Capto adhere	HiTrap Capto adhere	50 mM sodium phosphate pH 6.95, 6.75, and 6.55

Table 2. Design layout of the DoE for screening of loading conditions and results from the screening

DoE no.	Run order no.	Load (mg/ml medium)	Load cond. (mS/cm)	Load pH	Recovery (%)	HCP (ppm)	Protein A (ppm)	D/A (%)
DoE 1	8	100	10	6.0	82.3	9	< 1	0.93
DoE 2	1	301	10	6.0	98.0	31	< 1	0.67
DoE 3	10	104	30	6.0	72.6	10	< 1	0.65
DoE 4	7	312	30	6.0	96.4	16	< 1	0.86
DoE 5	6	93	10	7.5	49.0*	8	< 1	0.22
DoE 6	3	282	10	7.5	92.9	26	< 1	0.65
DoE 7	11	102	30	7.5	70.3	10	< 1	0.49
DoE 8	9	307	30	7.5	93.2	22	< 1	1.04
DoE 9†	5	192	20	6.75	92.2	13	< 1	0.86
DoE 10†	4	192	20	6.75	95.1	13	< 1	0.81
DoE 11†	2	192	20	6.75	93.4	12	< 1	0.69

* DoE 5 excluded, deemed an outlier
† DoE 9–11 were the center points in the design (load 192 mg/ml, conductivity 20 mS/cm, and pH 6.75)

Table 3. Design layout of the DoE for robustness of loading conditions and results from the robustness study

DoE no.	Run order no.	Load (mg/ml medium)	Load cond. (mS/cm)	Load pH	Chromatography medium lot	Sample Feed	Recovery (%)	HCP (ppm)	Protein A (ppm)	D/A (%)
DoE 1	2	190	17	6.55	A	Feed 2	86.7	20	< 1	2.3
DoE 2	9	210	13	6.55	A	Feed 1	93.9	55	2	1.2
DoE 3	3	190	17	6.95	A	Feed 1	76.1	25	< 1	1.7
DoE 4	10	210	13	6.95	A	Feed 2	83.7	55	2	1.5
DoE 5	4	190	13	6.55	B	Feed 2	86.4	25	< 1	2.1
DoE 6	5	210	17	6.55	B	Feed 1	85.3	55	2	1.3
DoE 7	7	190	13	6.95	B	Feed 1	78.1	15	< 1	1.9
DoE 8	6	210	17	6.95	B	Feed 2	88.3	25	< 1	2.2
DoE 9*	1	200	15	6.75	A	Feed 1	81.9	20	< 1	2.5
DoE 10*	8	200	15	6.75	A	Feed 1	85.4	55	1	1.7
DoE 11*	11	200	15	6.75	A	Feed 1	83.2	25	< 1	2.4

* DoE 9–11 were the center points in the design (load 200 mg/ml medium, conductivity 15 mS/cm, and pH 6.75)

The elution behavior of the MAb is defined by the pH at peak maximum (approximately 5.79) and is normally used to define the lower pH in such a design. However, in this case, precipitation in the sample occurred at low pH and low conductivity (< 10 mS/cm), and for that reason the lower pH in the design was set to pH 6.0 while conductivity was maintained at ≥ 10 mS/cm. The upper pH in the design would normally be chosen about 2 pH units above the elution pH, and was in this case set to pH 7.5. Conductivity varied between 10 and 30 mS/cm and load between 100 and 300 mg MAb/ml medium.

Robustness study on Capto adhere

A robustness study was performed by varying pH, conductivity, and load around the conditions established from the screening DoE. In addition, two different feeds and two different media lots were included.

Both feeds had higher levels of contaminants than the feed used in the screening DoE. A reduced design with five parameters, that is 8 experiments and 3 center points totaling 11 experiments, was set up (Table 3). The starting material concentration for the two feeds varied between 12.7 and 14.7 mg MAb/ml, HCP was 80 ppm for both feeds. Protein A was 3 ppm for feed 1 and 4 ppm for feed 2. D/A averaged 5.3% for feed 1 and 3% for feed 2. DoE 9–11 were the center points in the design (load 200 mg/ml medium, conductivity 15 mS/cm, pH 6.75).

Measurement of yield, HCP clearance, aggregate content, and protein A leakage

Determination of recovery and concentration

Concentration determinations were made either by measuring UV at 280 nm, using an extinction coefficient of 1.49, or by affinity chromatography using a HiTrap MabSelect SuRe 1 ml column on ÄKTAexplorer™ 10 chromatography system.

Analysis of dimer/aggregate content

Flowthrough fractions were analyzed by gel filtration (size-exclusion chromatography) using two interconnected Superdex™ 200 5/150 GL columns connected to ÄKTAexplorer 10 system (data not shown). An aliquot of each sample was applied to the column and run in PBS at a flow rate of 0.35 ml/min for 15 min. Aggregate concentration determinations were made by measuring UV at 280 nm and calculating peak area ratios.

Analysis of host cell protein (HCP) and ligand leakage

HCP levels were measured using commercial anti-CHO HCP antibodies (Cygnus Technologies Inc.). Essentially, an ELISA methodology was adapted to Gyrolab™ Workstation LIF (Gyros AB, Uppsala, Sweden) using Gyrolab Bioaffy™ 20 HC microlaboratory discs. Ligand leakage (MabSelect SuRe protein A ligand) measurements were performed using a commercial ELISA kit (Repligen Corp., Waltham, MA, USA) with a modified protocol.

SDS-PAGE analysis

Samples were adjusted to pH 8.5 with 1 M NaOH. A working solution of CyDye™ DIGE Fluor minimal dye (Cy5™) was prepared in dimethylformamide (5 nmol CyDye to 12.5 µl dimethylformamide). The Cy5 solution (1 µl) was added to 50 µg protein followed by incubation for 30 min in the dark on an ice bath. The reaction was stopped with 1 µl of 10 mM lysine. Unreduced samples (5 µg/well) were run on Multiphor™ II flatbed electrophoresis system using precast ExcelGel™ SDS Gradient 8–18 gels. These gels were scanned using Ettan™ DIGE Imager. This was performed at several different PMT (photomultiplier tube) voltages on account of the significant difference in amount of MAb and amount of contaminant.

Results and discussion

The two-step purification developed consisted of a protein A-based capture step and multimodal anion exchange polishing step. Using ÄKTA avant 25 system controlled by UNICORN 6, process development was achieved in approximately one week. The method developed allowed high recovery of a high purity MAb. The purity achieved could be considered suitable for most therapeutic use but could also be improved using a three-step purification.

The material used was challenging, primarily due to precipitation of the sample that necessitated running the second step at elevated conductivity.

Lower conductivity will normally result in better contaminant removal (1). Another challenging aspect was the increased aggregation of MAb over time and at low pH or conductivity, thus sample used for the first experiments was lower in D/A than sample used in later experiments, for example, in the robustness study.

Capture of MAb using MabSelect SuRe

Determination of elution pH and dynamic binding capacity on MabSelect SuRe

Elution of the MAb was performed in a linear pH gradient as shown in Figure 4. The elution pH was 3.67 at peak maximum and 3.56 at 10% of peak maximum (i.e., descending). Based on this result, it was decided to use an elution pH of 3.5, which would result in high yields and narrow elution peaks. The dynamic binding capacity at 10% breakthrough (DBC 10%) was determined to be 21 and 40 mg/ml at residence times of 2.4 and 4 min, respectively.

Column:	HiScreen MabSelect SuRe, 4.7 ml
Sample:	1 ml of clarified CHO feed containing 1.72 mg MAb/ml
Binding buffer:	20 mM citrate, pH 6.0
Elution buffer:	20 mM citrate, pH 3.0
Gradient:	Linear, 20 mM citrate buffer pH 6.0 to 3.0 in 10 CV
Flow rate:	0.5 ml/min
System:	ÄKTA avant 25

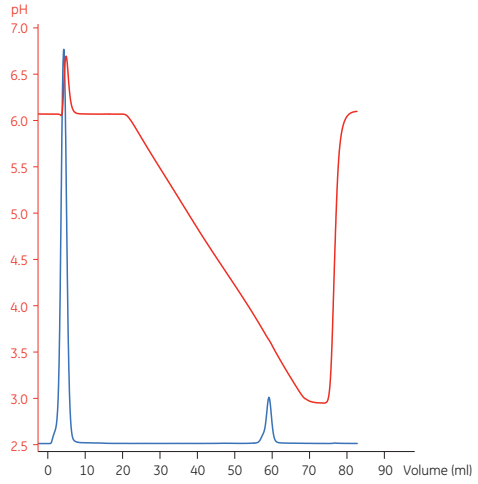


Fig 4. Determination of elution pH on HiScreen MabSelect SuRe.

Purification of Mab on MabSelect SuRe

An example of a chromatogram from purification of the MAb on a HiScreen MabSelect SuRe column is shown in Figure 5. Recovery of 99% was achieved, with HCP at 25 ppm, 0.8% D/A, and leached protein A at 6 ppm.

Column:	HiScreen MabSelect SuRe, 4.7 ml
Sample:	112.5 ml of clarified CHO feed containing 1.17 mg MAb/ml
Binding buffer:	PBS, pH 7.4
Elution buffer:	50 mM citrate, pH 3.5
Flow rate:	1.2 ml/min, residence time 4 min
Gradient:	Single step, from binding to elution buffer
Cleaning-in-Place (CIP):	0.1 M NaOH in 2 CV, contact time 15 min
System:	ÄKTA avant 25

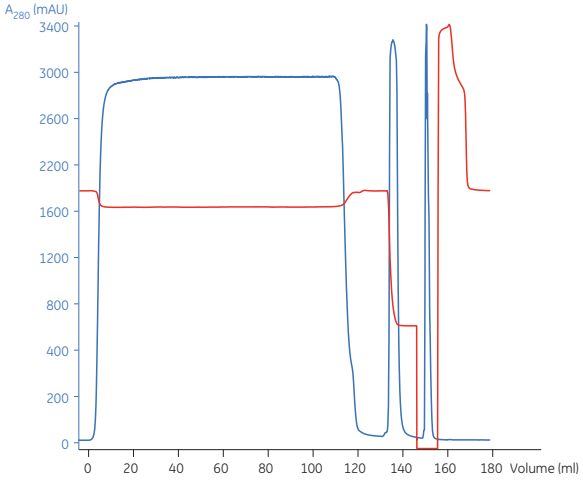


Fig 5. Purification of MAb on HiScreen MabSelect SuRe. The first peak after the flowthrough is the purified MAb, while the second peak contained contaminants from the CIP. The apparent drop in pH (red curve) was a result of bypassing the pH monitor while applying NaOH, and is therefore not a true measure of pH at this point of the purification run.

Scale-up on XK 50/20 column prior to Capto adhere polishing step

The method was scaled up to an XK 50/20 column for preparation of material for the polishing-step experiments (data not shown). ÄKTAexplorer 100 system was used for the scale-up. Purified antibody pools from the XK 50/20 runs were then used for screening- and robustness-runs on Capto adhere. Levels of HCP, protein A, and D/A were somewhat higher in the XK 50/20 scale-up than during the previous runs.

Polishing step using Capto adhere

Initial screening of binding and elution conditions

Three milligram of MAb was loaded on a HiScreen Capto adhere column at pH 7.8. Elution was performed with a linear pH gradient from pH 7.8 to 4.0. The MAb eluted in a relatively broad peak (Fig 6). Elution pH at peak maximum was 5.79. The elution position defines the lower pH in the design for further optimization. However, due to precipitation at low pH and conductivity, the lower pH in the design was set to 6.0 and conductivity was maintained above ≥ 10 mS/cm.

Column:	HiScreen Capto adhere, 4.7 ml
Sample:	1 ml elution pool from HiScreen MabSelect SuRe diluted in start buffer to 3 mg/ml
Start buffer:	20 mM sodium phosphate, 20 mM citrate, pH 7.8
Elution buffer:	20 mM sodium phosphate, 20 mM citrate, pH 4.0
Gradient:	Linear, 20 mM phosphate, 20 mM citrate, pH 7.8–4.0 in 10 CV
Flow rate:	2.4 ml/min
System:	ÄKTA avant 25

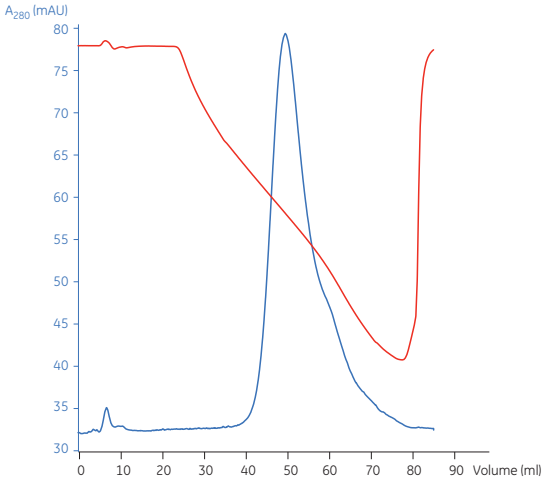


Fig 6. Determination of binding and elution conditions on HiScreen Capto adhere.

Design of Experiments for screening of loading conditions

A representative, typical flowthrough chromatogram (whereby MAb elutes in flowthrough) from the screening design using HiTrap Capto adhere is shown in Figure 7 and the data from the design are summarized in Table 2. The chromatogram is representative of a center point DoE for screening (Table 2) .

Column:	HiTrap Capto adhere, 1 ml
Sample:	Elution pool from HiScreen MabSelect SuRe (center point in design, i.e. pH 6.75, conductivity 15 mS/cm, and load 200 mg/ml medium)
Start buffer:	20 mM sodium phosphate, 20 mM citrate, pH 6.75 Conductivity adjusted to 15 mS/cm with NaCl
Regeneration buffer:	0.1 M acetate, pH 3.0.
Gradient:	Step gradient between start buffer, regeneration buffer, and CIP
Flow rate:	2.0 ml/min
CIP:	1 M NaOH, contact time 15 min
System:	ÄKTA avant 25

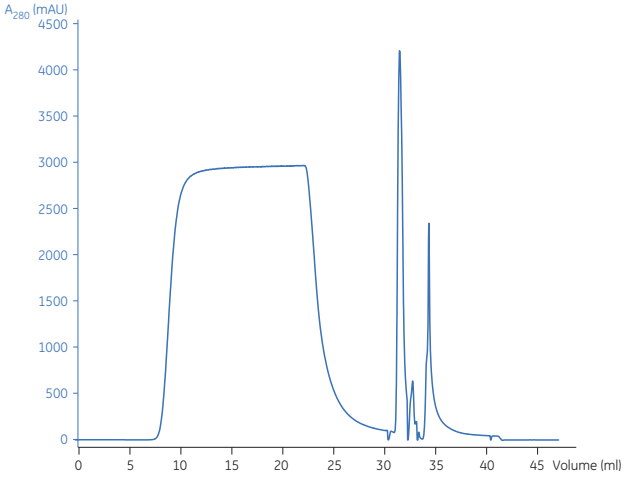


Fig 7. Chromatogram from HiTrap Capto adhere screening design. MAb eluted in the flowthrough while the two peaks at approx. 32 ml and 35 ml were aggregates and impurities from the CIP, respectively.

Figure 8 summarizes the results from the screening DoE. Results are visualized in three different ways, by using "Summary of fit", "Coefficient plot", and "Contour plots". Three responses were analyzed: recovery, HCP removal, and aggregate (D/A) removal. The fourth response, protein A leakage, was too low to allow design of a statistical model. In fact, the protein A leakage observed in eluted fractions from the HiScreen MabSelect SuRe column was already very low.

In the Summary of fit plot, percent variation of the response explained by the model (R2) > 0.8 for both the recovery and aggregate models indicating a good model fit. A high predictive power (percent variation in response predicted by the model according to cross-validation, Q2) was obtained in the model (> 0.5) for both of these factors. Model validity values indicate strong models with values > 0.5 and excellent models above 0.9. For HCP removal, a model of moderate quality and low validity was obtained. Reproducibility, the measure of replicate variation obtained for this model, was > 0.5 indicating minimal error and good control of the experimental procedures.

The Coefficient plots display regression coefficients with confidence intervals. The significant coefficients remained in the model, as well as some insignificant coefficients if an interaction term was found to be significant.

The Contour plots show the effect of two factors on the specific responses.

As expected, increased load positively influenced recovery while contaminant clearance was improved at lower load.

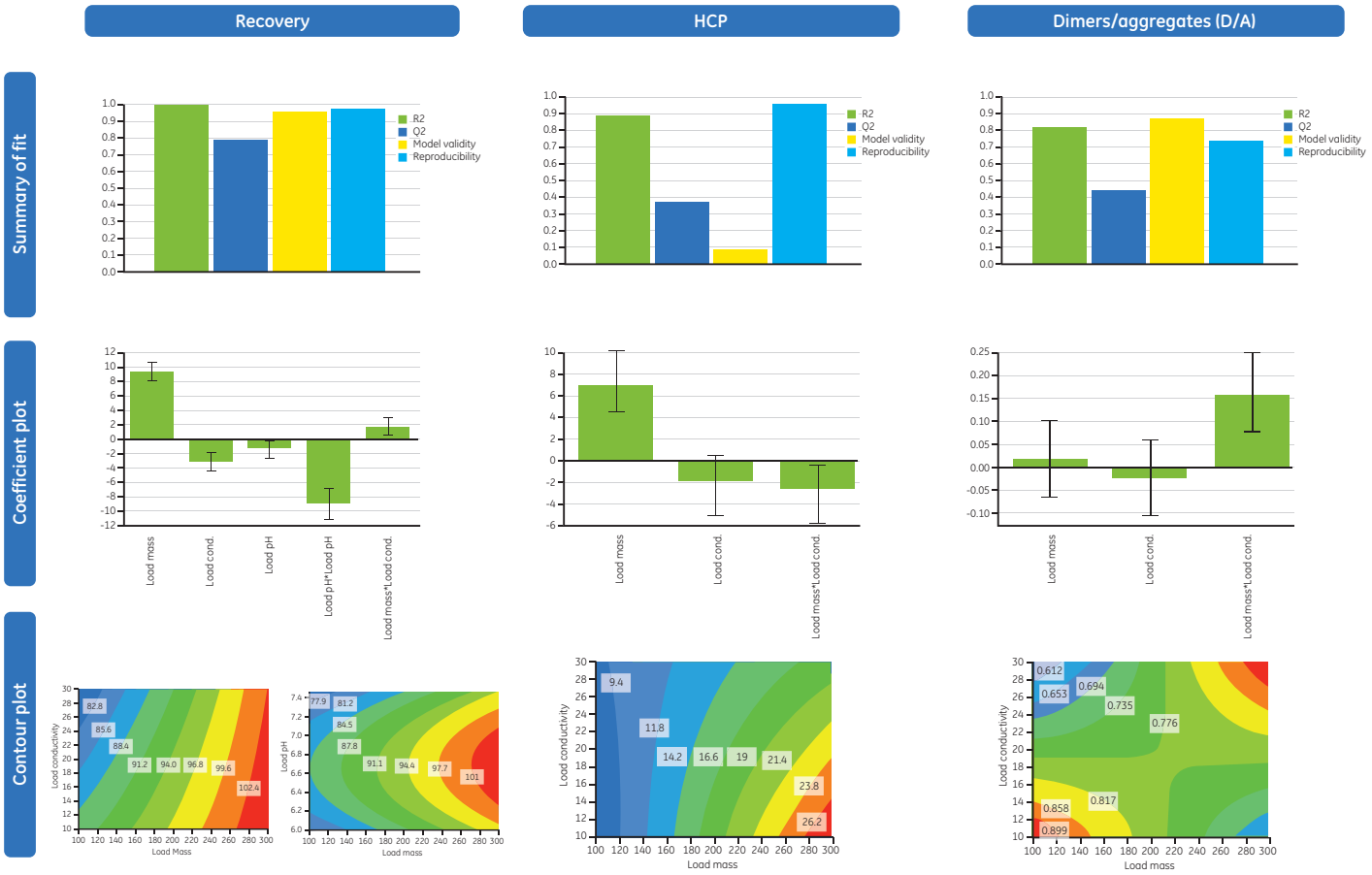


Fig 8. DoE results for the screening of conditions for the Capto adhere step. The three responses analyzed separately were recovery, HCP, and D/A. The results are visualized in three different ways: "Summary of fit", "Coefficient plot", and "Contour plots".

Robustness study on Capto adhere

The results are summarized in Table 3. Figure 9 shows SDS-PAGE analysis of the individual DoE runs and the two different feeds used, and Figure 10 shows a "Summary of fit" plot from the robustness study. No model could be built from the obtained results, thus the conditions were deemed to be robust. This view is supported by the SDS-PAGE results where no obvious differences between the 11 DoE lanes/runs can be seen (Fig 9).

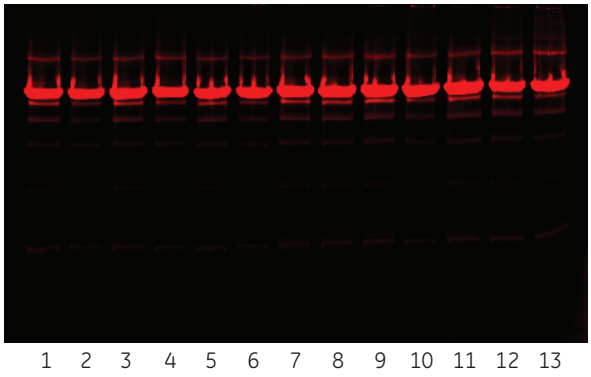


Fig 9. SDS-PAGE of individual runs in the robustness DoE, run on ExcelGel SDS Gradient 8–18 under nonreducing conditions. The gel is visualized by Cy5. Lanes 1–11 correspond to DoE experiment numbers 1–11, respectively. Lanes 12–13 are feeds 1 and 2, respectively.

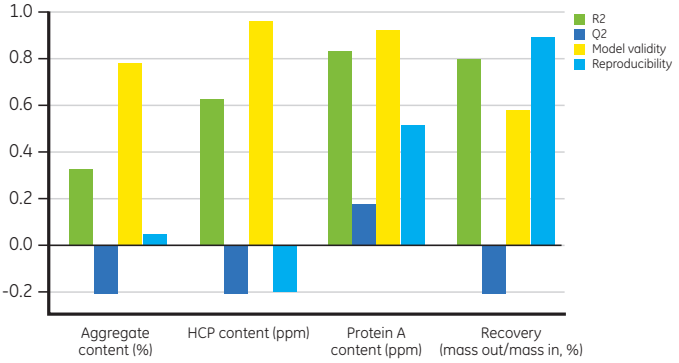


Fig 10. Summary of fit from the DoE robustness study. Results for D/A, HCP, protein A content, and recovery.

Conclusions

A two-step chromatography process was developed using ÄKTA avant 25 chromatography system and UNICORN 6 software in combination with prepacked HiTrap and HiScreen columns in approximately one week. With the unique selectivity and generic method conditions possible with ÄKTA avant 25, UNICORN 6, MabSelect SuRe, and Capto adhere prepacked columns, straightforward method optimization was achieved. The flexible design of ÄKTA avant 25 system allows modification of the standard flow configuration; in this study, two sample inlet valves were used to enable automated sample loading and wash without loss of sample. Runs were performed using the novel pressure/flow regulation feature of the system, which ensured that pressure buildup during sample loading was minimized. The DoE approach was highly useful for screening and optimization of conditions for the Capto adhere polishing step. The DoE module in UNICORN 6 software facilitates a quick setup of run schemes for determining optimal and robust conditions, enabling very high productivity.

References

1. Application note: Optimization of loading conditions on Capto adhere using Design of Experiments, 28-9078-89 Edition AA, GE Healthcare.
2. Eriksson, K. *et al.* MAb contaminant removal with a multimodal anion exchanger. A platform step to follow protein A. *BioProcess International,* **7 (2)** 52–56 (2009).

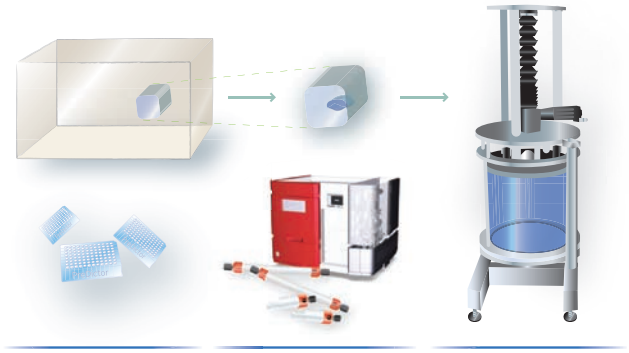
Ordering information

Product	Code no.
ÄKTA avant 25 chromatography system	28-9308-42
UNICORN 6 local and remote workstation license with DVD	28-9589-93
UNICORN 6 local and remote workstation license without DVD (one additional license)	28-9589-95
HiTrap MabSelect SuRe, 5 × 1 ml	11-0034-93
HiTrap Capto adhere, 5 × 1 ml	28-4058-44
HiScreen MabSelect SuRe, 1 × 4.7 ml	28-9269-77
HiScreen Capto adhere, 1 × 4.7 ml	28-9269-81
Superdex 200 5/150 GL, 1 × 3 ml	28-9065-61
Cy5 DIGE Fluor minimal dye, 5 nmol	25-8008-62
XK 50/20 column, 50 mm i.d. × 18 cm	18-1000-71
ExcelGel SDS Gradient 8–18, 6 precast gels	80-1255-53
Multiphor II electrophoresis system	18-1018-06
Ettan DIGE Imager	63-0056-42

GE Healthcare process development workflow

GE Healthcare's BioProcess™ systems, chromatography media, columns, and 96-well plates cover all purification steps from capture to polishing, and cover all scales of work from development and pilot studies to routine production. ÄKTA avant 25 has flow rate and pressure specifications that support BioProcess media such as MabSelect and Capto. These BioProcess media provide increased dynamic binding capacity at high flow rates. Using ÄKTA avant 25 together with UNICORN control software and high-flow BioProcess media reduces process time, increases productivity, and ensures easy scale-up.

All media are manufactured using validated methods and tested to meet stringent quality requirements.



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