Polishing of monoclonal antibodies using Capto MMC ImpRes in bind and elute mode

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Polishing of monoclonal antibodies using Capto™ MMC ImpRes in bind and elute mode

A rapid procedure to establish a robust second step in bind/elute (B/E) mode for the purification of a MAb using Capto MMC ImpRes has been developed. This study presents results from optimization of the elution conditions using the Design of Experiments (DoE) approach. The results show high yield of monomeric MAb, as well as good clearance of aggregates, host cell proteins (HCP), and leached protein A.

Introduction

GE Healthcare Life Sciences´ MAb production toolbox employs protein A chromatography media (resins) such as MabSelect SuRe™ or MabSelect SuRe LX for capture of the target. After the initial protein A step, there is a range of options for intermediate and polishing purification steps. One of these options, Capto MMC ImpRes, is a cost-effective and flexible chromatography medium designed for high-resolution polishing of MAbs. Capto MMC ImpRes complements GE Healthcare's MAb toolbox by providing a polishing medium with the advantages of multimodal selectivity that gives high yields of MAb and good clearance of impurities.

Capto MMC ImpRes is a chromatographic medium based on a multimodal cation exchange ligand (Fig 1). The ligand constitutes a hydrophobic part, a weak cation exchange group, and groups that can promote hydrogen bonds. Capto MMC ImpRes comprises small (~ 40 µm) beads relative to the related multimodal cation exchange medium, Capto MMC (~ 75 µm).

In order to fine-tune the protein/ligand interaction for optimal aggregate removal, the ligand density of Capto MMC ImpRes has been reduced significantly (25 to 39 µmol/mL) compared to Capto MMC (70 to 90 µmol/mL). The effect of this is improved selectivity between monomer and aggregates

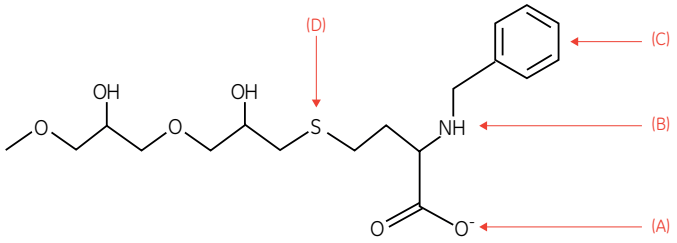

Fig 1. The multimodal ligand of Capto MMC adhere; N-benzoyl-homocysteine. The ligand exhibits multimodal functionality for interaction with a target molecule. The most pronounced of these interactions are ionic (A), hydrogen bonding (B), hydrophobic interactions (C), and thiophilic interactions (D). The chromatography medium is designed for polishing, and is based on the high-flow agarose base matrix with small bead size, which gives good pressure-flow properties and high resolution.

compared to Capto MMC. Another effect of the lower ligand density is reduced salt tolerance. This simplifies elution from Capto MMC ImpRes with salt leading to higher yield and smaller pool volumes. The medium still has a higher salt tolerance than traditional cation exchangers, which enables loading at moderate levels of salt, that is, direct loading after the protein A step without dilution.

This Application note describes a fast and efficient method to separate monomeric MAb from aggregates, HCP, and protein A remnants. The method described includes screening for optimal binding conditions in 96-well plate format followed by verification in packed column format and optimization of elution conditions using Design of Experiments (DoE). The running conditions were then validated in larger scale with satisfactory correspondence to DoE model prediction. All preparative chromatography experiments were performed in B/E mode.

A summary of the steps in the study is shown in Figure 2.

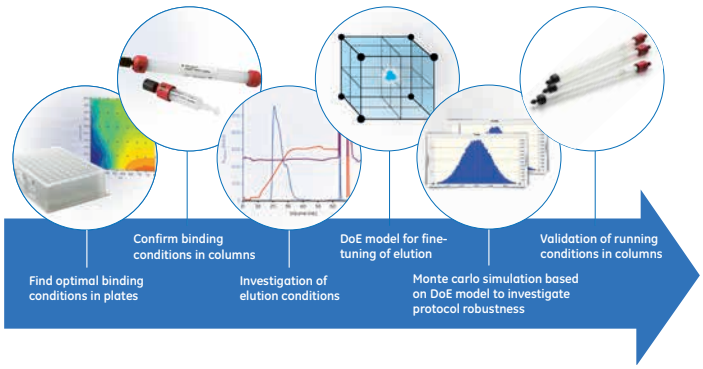

Fig 2. The steps used in this performance evaluation of Capto MMC ImpRes in removing contaminants from monomeric MAb.

Materials and methods

Start material

The MAb used in this study was initially purified from CHO cell supernatant by protein A affinity chromatography. Some characteristics of the antibody are shown in Table 1.

Table 1. Characteristics of the antibody used in the study

Antibody	pI	Aggregate content (%)	DBC 10% (mg/mL)* Capto MMC ImpRes
MAb A	7.3	2.5	71

* Dynamic binding capacity (DBC) at 10% breakthrough (DBC 10%) measured at 4 min residence time (RT).

Determination of static binding capacity

Static binding capacity (SBC) was determined in 6 µL PreDictor™ Capto MMC ImpRes 96-well plates. Equilibration of wells in the plates was performed by addition of 200 µL of loading buffer per well followed by agitation at 1100 rpm for 1 min, after which the buffer was removed by vacuum suction. The equilibration step was performed three times. MAb, partially purified by protein A affinity chromatography (200 µL volume, 4 mg/mL sample load, corresponding to 133 mg MAb/mL chromatography medium) was added to each well followed by agitation for 90 min. Unbound material (FT fraction) was removed by centrifugation for 1 min, and MAb concentration was determined by measurement of absorbance at 280 nm.

SBC was calculated according to:

$$MAb_{bound} = 0.2 \times (C_{in} - C_{out})\ [\text{mL} \times \text{mg/mL} = \text{mg}]$$

$$SBC = MAb_{bound}/V_{medium} = MAb_{bound}/0.006\ [\text{mg/mL}]$$

where C_{int} = MAb concentration in sample, C_{out} = MAb concentration in FT fraction, and V_{medium} = medium volume in each well (i.e., 6 µL).

Determination of dynamic binding capacity

Dynamic binding capacity (DBC) was determined by frontal analysis using an ÄKTAexplorer™ 10 chromatography system. The UV-absorbance at 280 nm was used for determination of breakthrough. The dynamic binding capacity was then calculated according to:

$$DBC_{x\%} = (V_{x\%} - V_0) * C_0/V_c$$

were $V_{x\%}$ = load volume (mL) at x% breakthrough, V_0 = void volume (mL), C_0 = MAb concentration in the sample (mg/mL) and V_c = volumetric bed volume (mL).

Screening of elution conditions

Conditions for optimizing elution were investigated in a Tricorn™ 5/50 packed column with Capto MMC ImpRes at a bed height of 4.7 cm. Optimization was performed using ÄKTA™ avant 25 chromatography system, DoE, and scouting functionalities included in UNICORN™ 6.0 software. The factors considered in the design were load volume, gradient length, and flow velocity. The responses were resolution of monomer/aggregates and pool volume.

The method used for the DoE runs was the following:

Column:	Tricorn 5/50, bed height 4.7 cm
Medium:	Capto MMC ImpRes
Sample:	MAb (8 mg/mL) equilibrated in start buffer
Start buffer:	25 mM sodium phosphate, 25 mM sodium citrate, 100 mM NaCl, pH 6.0
Elution buffer:	Start buffer + 1 M NaCl
Wash:	Start buffer (5 CV)
CIP:	1 M NaOH

Determination of MAb aggregates and aggregate clearance

Fractions from the chromatographic runs were collected and analyzed by gel filtration (analytical size exclusion chromatography) on a Superdex™ 200 5/150 GL column. The peaks were integrated and the dimer/aggregate content (in percent) were estimated. Cumulated yield of monomers was plotted against cumulated aggregates (Fig 3).

Protein A and HCP ELISA

The protein A concentration in the start materials and flowthrough fractions was determined by Protein A ELISA kit (Repligen). HCP concentration was determined by HCP ELISA (Cygnus Technologies).

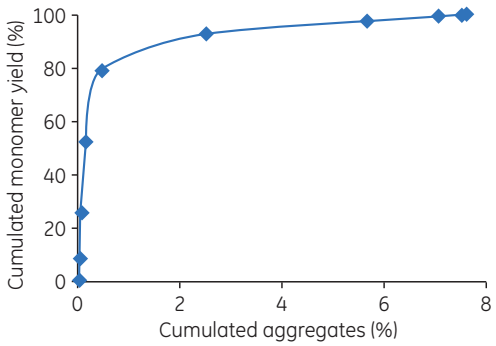

Fig 3. Evaluation of gradient elution was performed by gel filtration. The Figure shows an example of the resulting plot of cumulated yield of monomers vs cumulated aggregates derived from the gel filtration analysis.

Results and discussion

Case study

The case study with MAb shows a suggested workflow for method development including screening of binding conditions in 96-well format, verification of dynamic binding capacities in column format, screening-, optimization of elution conditions, and validation of the DoE model prediction in a HiScreen column. It also includes a Monte Carlo simulation that addresses the protocol robustness.

Static binding capacity

To find optimal binding capacity for the MAb, static binding capacity (SBC) was determined in 6 µL PreDictor 96-well filter plates. Binding pH was varied between pH 4.0 and 8.0[1,2] and salt concentration from 0 to 500 mM NaCl. All samples and buffers were prepared automatically using an automatic liquid handling system for the preparation of buffers. The results from the SBC study display an area of conditions with high binding capacities between pH 5.0 and 7.0 and NaCl between 0 and 150 mM. The highest SBC was obtained at approx. pH 6.0 and salt concentration of 0 to 150 mM (Fig 4). The binding capacity appeared to be more salt tolerant at lower pH and could represent an alternative binding condition. This is important to take into account since the choice of binding conditions affects the elution strategy. When binding at pH between 6.0 and 6.5, elution can be performed merely using a salt gradient whereas binding at pH between 5.0 and 5.5 is likely to require salt and pH gradient elution.

[1] Start buffers were sodium acetate, pH 4.0 and 5.3; sodium phosphate, pH 6.3; Tris, pH 8.0.

[2] To avoid deamidation of the MAb, pH should normally be maintained below pH 8.0.

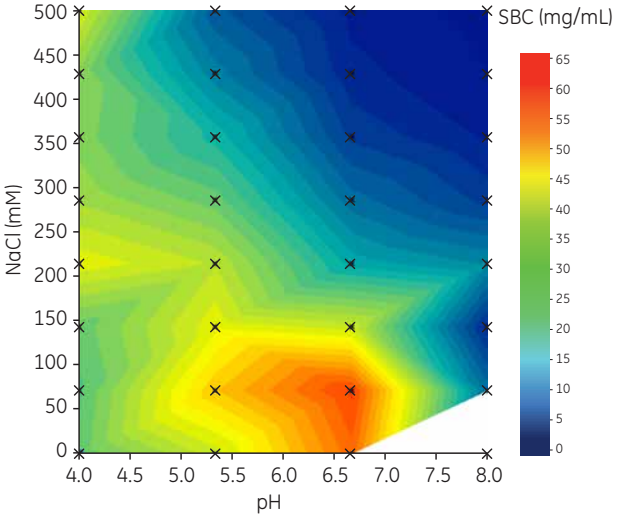

Fig 4. Contour map showing screening of SBC for Capto MMC ImpRes. The lower right corner is excluded since MAb tended to precipitate at pH > 6.7 and low salt concentration.

Dynamic binding capacity

The area with high SBC in the PreDictor plate studies—between pH 5.0 and 6.0 and NaCl content between 0 and 200 mM—were chosen for further investigation in column format. This particular MAb showed a tendency to precipitate at pH > 6.7 at certain conditions, which explains the choice of pH values for verification.

The trends seen in the PreDictor plate SBC experiment correlated well with the DBC studies (Fig 5). The condition with highest DBC (100 mM NaCl, pH 6.0) was chosen for further investigation in which the influence of residence time was studied (Fig 6). The DBC was found to be relatively independent of residence time in the investigated interval.

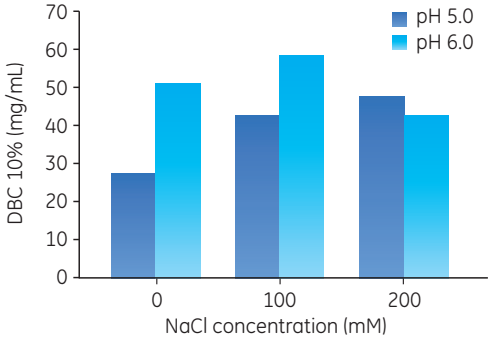

Fig 5. Dynamic binding capacity of Capto MMC ImpRes at 4 min RT in different salt concentrations and two different buffer pH.

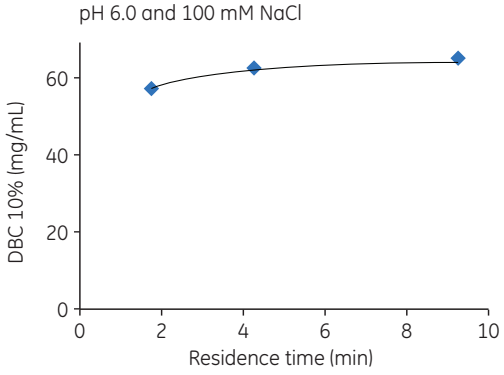

Fig 6. Influence of residence time on DBC measured at 10% breakthrough.

Investigating elution conditions for selectivity

As high binding capacities were found at pH 5.0 to 7.0, aggregate removal and yield were investigated by linear NaCl gradient elution at pH 5.0, 6.0, and 7.0 (Fig 7).

Column:	Tricorn 5/50, bed height 4.7 cm
Medium:	Capto MMC ImpRes
Sample:	4 mL of MAb (6.3 mg/mL)
Start buffer stock solution:	BufferPro CIEX 2–7 (sodium phosphate, sodium formate, sodium acetate buffer, various pH)
Start buffer A:	pH 5.0
Start buffer B:	pH 6.0
Start buffer C:	pH 7.0
Elution buffer:	Start buffer + 1 M NaCl
Wash:	Start buffer, 5 column volumes (CV)
Gradient:	0% to 100% elution buffer in 20 CV
Residence time:	4 min
CIP:	1 M NaOH
System:	ÄKTA avant 25

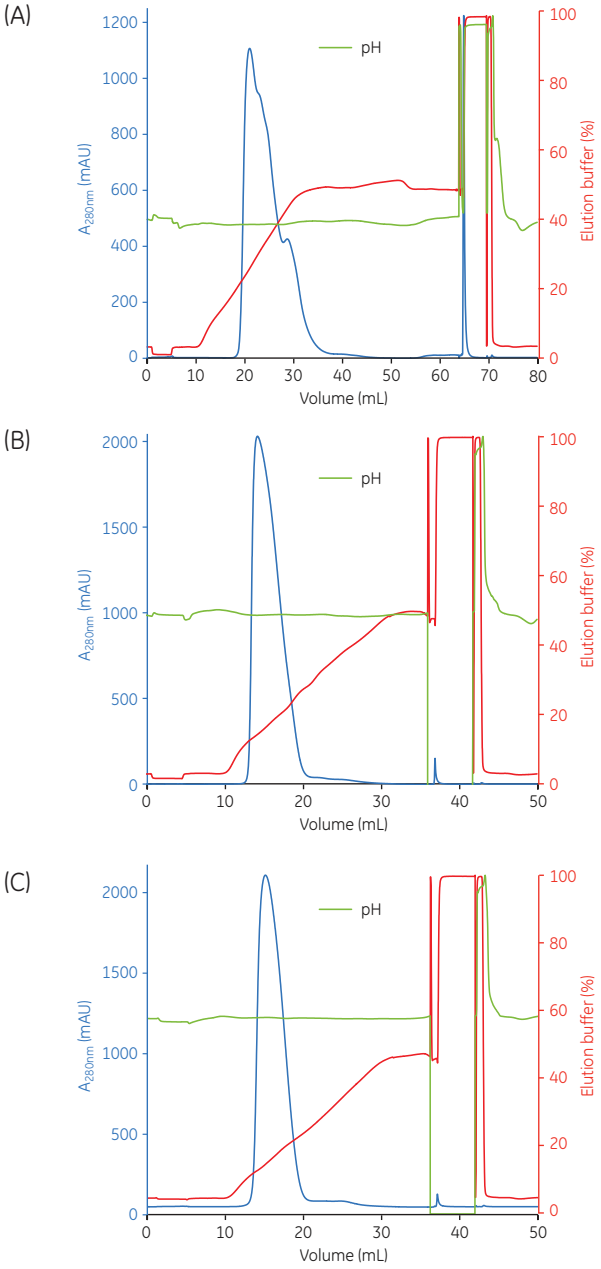

Fig 7. Elution of the MAb in a salt gradient at three different pH: (A) pH 5.0, (B) pH 6.0, and (C) pH 7.0.

Fractions were collected and analyzed by gel filtration and fractions containing 90% of the MAb were pooled and analyzed for HCP and protein A content. A summary of the results is found in Table 2. As can be seen, efficient aggregate removal at 90% yield was obtained for all three binding pH values. However, at pH 5.0, larger pool volumes were observed and precipitation tendencies were seen for pH 7.0. Therefore, the conditions chosen were binding at pH 6.0 and elution with an NaCl gradient.

If higher purity levels or higher yield at maintained purity had been required than the performance observed, a pH closer to 5.0 for binding and NaCl gradient elution would be a suitable alternative.

Table 2. Summary of the results of the chromatography at different pH; start concentrations for HCP and protein A are shown in brackets

pH	Aggregate at 90% yield (%)	Pool volume (CV)	HCP (ng/mL)	Protein A (ng/mL)
5.0	0.04	14.1	16 (245)	Below LOQ* (16)
6.0	0.2	5.4	56 (245)	Below LOQ (16)
7.0	0.2	6.5	44 (245)	Below LOQ (16)

* LOQ = Limit of quantitation

Optimization of the purification performance

The binding study showed that binding at pH 6.0 with an addition of 100 mM NaCl resulted in high binding capacities. Also high purity and yields were obtained at pH 6.0 using a linear salt gradient for elution. It would be possible at this point to stop further evaluation, but in order to build understanding and to optimize the purification performance, a DoE model was set up for the influence of three factors on aggregate content and pool volume. The factors that were varied and responses are displayed in Table 3. The reduction of HCP and protein A was not included as a response in the design but was measured. The start concentration of HCP was 164 ng/mL and the start concentration of protein A was 26 ng/mL. Other factors that affect the purification performance and could be of interest to study from a robustness perspective using this methodology are for example aggregate content or HCP levels.

The rationale behind the high and low levels of the parameters in the DoE model was as follows; flow velocity was chosen to ensure that high and low flow velocities corresponded to a residence time (RT) of 2 and 8 min, respectively. For many processes, it is not possible to have shorter RT than 2 min due to limitations in the pumps and other equipment. The low flow velocity gives a longer RT but is still acceptable.

Gradient length was between 5 and 15 CV. The short gradient length of 5 CV challenged the performance of Capto MMC ImpRes since this gradient is shorter than most gradients used in purification processes today. A gradient length of 15 CV is closer to that typically used by process developers and represents an average, normal gradient length.

In this study, loads of 42 and 30 mg sample/mL were used. These represent loading of 70% and 50%, respectively of the DBC 10%. A loading of 70% of DBC 10% is usually regarded as the upper limit for loading without risking any leakage of target molecules. A loading of 50% of DBC 10% is substantially lower, but represents a plausible loading for a process.

Table 3. Factors and levels studied in by DoE

Factors	Low	High
Load (mg/mL MAb)	30	42
Gradient length (CV)	5	15
Flow velocity (cm/h)	37	140

Aggregate removal

Flow rate and gradient length were found to significantly affect aggregate removal while the influence of load was insignificant. Lower flow rates and longer gradients resulted in lower aggregate amounts. The significant factors in the model are shown in the coefficient plot (Fig 8A).

The summary plot in Figure 8B shows different model characteristics such as model fit (R^2), an estimate of the precision of future predictions, model validity, and information on the reproducibility. The summary plot indicates that the model is valid.

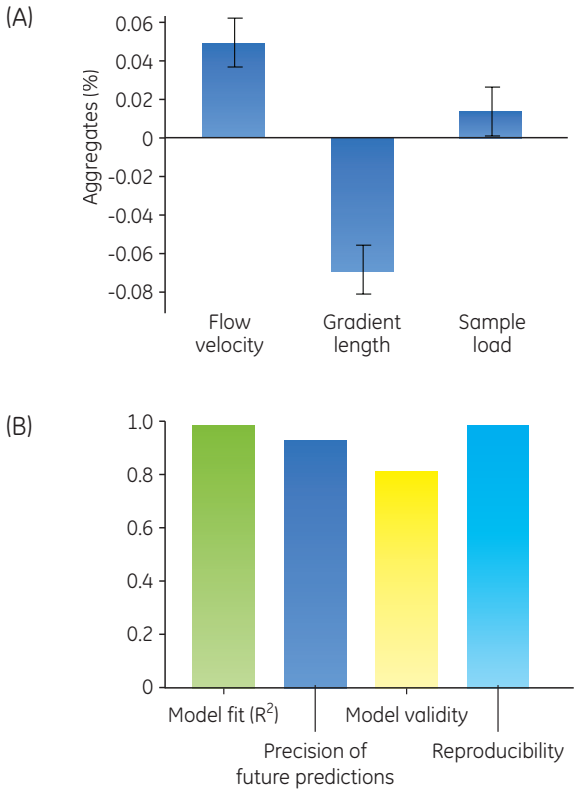

Fig 8. (A) Coefficient plot showing factors affecting the aggregate removal. (B) Summary plot showing different model characteristics for the aggregate removal response.

Table 4. Summary of the factors and responses used in the three-factor screening design

Factors			Responses			
Flow velocity (cm/h)	Gradient length (CV)	Sample load (mg/mL)	Pool volume (CV)	Aggregate at 90% yield (%)	HCP (ng/mL)	Protein A (ng/mL)
37	5	30	2.7	0.4	93	*
37	15	30	4.9	0.23	39	*
37	15	42	6.0	0.26	71	*
89	10	36	4.3	0.39	41	*
89	10	36	4.3	0.39	60	*
89	10	36	3.8	0.37	27	*
140	5	30	3.3	0.48	58	*
140	15	30	5.4	0.37	74	*
140	5	42	3.3	0.42	62	*
140	15	42	6.0	0.37	79	*

* Below LOQ

Pool volume

Gradient length and sample load were significant factors affecting pool volume (Fig 9). The summary plot in Figure 9B describes the characteristics and validity of the model.

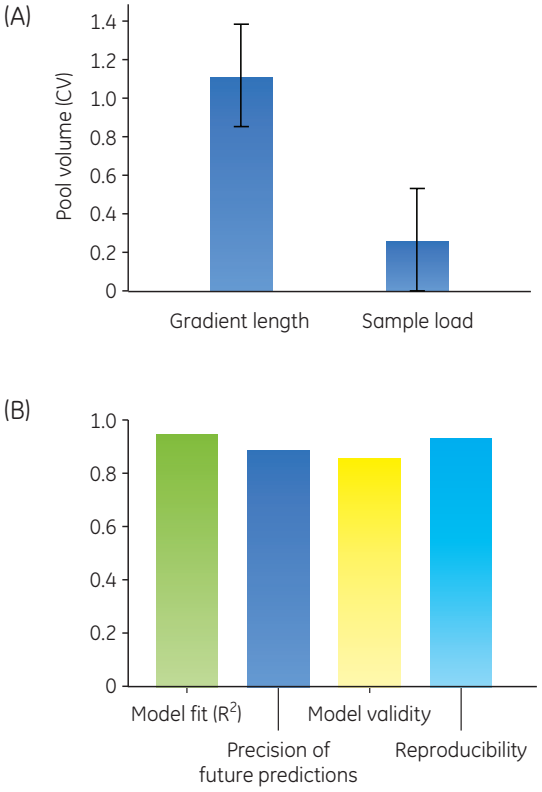

Fig 9. (A) Coefficient plot showing factors affecting pool volume. (B) Summary plot showing different model characteristics for the response pool volume.

Prediction of aggregate removal and pool volumes using DoE and Monte Carlo simulation

To find optimal parameters for a purification protocol and investigate the robustness of that protocol, a Monte Carlo simulation based on the DoE model was used. The investigated design space for the DoE model and the target purification performance are shown in Table 5. The suggested chromatographic protocol and the allowed variation in each factor (triangular distribution) are shown in Table 6. A Monte Carlo simulation was used in order to assess the design space with probabilities of failing to meet the target purification performance. The resulting design space defined by the Monte Carlo simulation is shown in Figure 10.

Table 5. Factors, responses, and target values for optimization in the DoE model

Factors		Low	High
Flow velocity (cm/h)		37	140
Gradient length (CV)		5	15
Load (mg/mL)		30	42
Response	**Criterion**	**Target**	**Max.**
Aggregate content (%)	Minimize	0.2	0.4
Pool volume (CV)	Minimize	2.0	4.5

Table 6. Factors, variation, and distribution of the factors of the final purification protocol used in the Monte Carlo simulation

Factors	Low	Optimum	High	Distribution
Flow velocity (cm/h)	46	49	52	Triangular
Gradient length (CV)	10	10.5	11	Triangular
Load (mg/mL)	31	34	36	Triangular

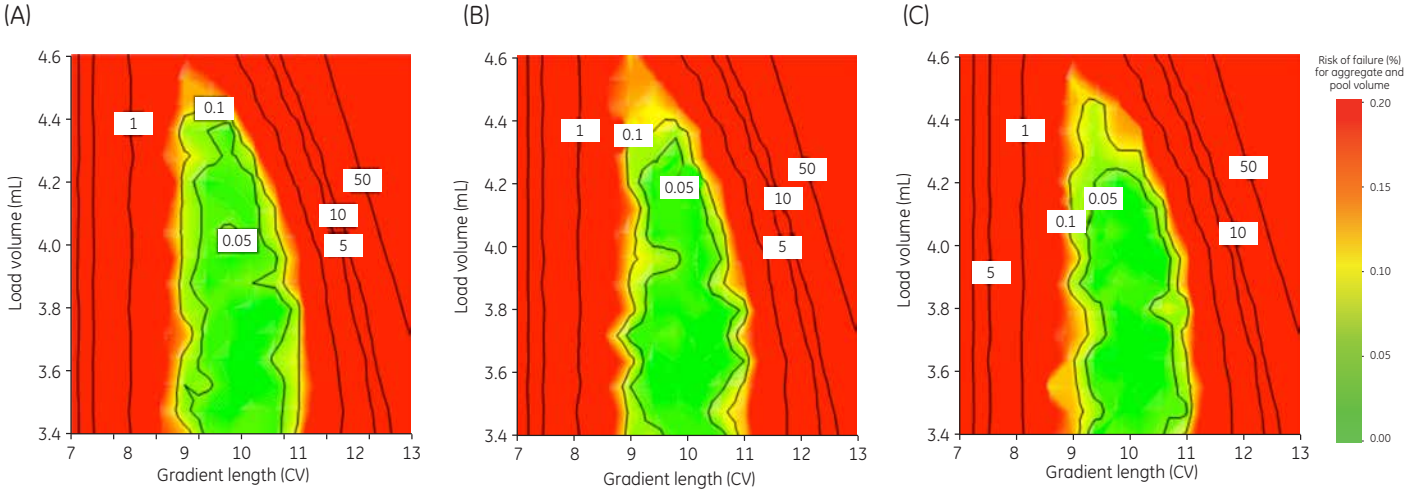

Fig 10. Contour plots from the Monte Carlo analysis showing risk of failing (red area), as well as running conditions to meet the desired purification performance (green area) at flow velocities of (A) 46, (B) 49, and (C) 52 cm/h.

Validation of the DoE model

To validate the model, running conditions that would fulfill the desired purification performance (Fig 10, green area) were chosen and applied to a 4.7 mL HiScreen™ Capto MMC ImpRes column on ÄKTA avant 25 chromatography system. Flow rate and load volume were recalculated according to the size of the HiScreen column (see Materials and methods). The chosen running conditions are summarized below and the purification performance was predicted using UNICORN 6.0 software. The factor settings selected for validation of the model are shown in Table 7.

Column: HiScreen Capto MMC ImpRes, 4.7 mL
Sample: 20.25 mL MAb A (34 mg/mL) in 25 mM sodium citrate +100 mM NaCl, pH 6.0
Start buffer: 25 mM sodium citrate + 100 mM NaCl, pH 6.0
Elution buffer: Start buffer + 1 M NaCl
Flow velocity: 49 cm/h
Gradient: 0% to 100% in 10.5 CV
System: ÄKTA avant 25

Table 7. Factors selected for validation of the model

Flow velocity (cm/h)	Gradient length (CV)	Sample load (mg/mL)
49 cm/h	10.5	34

Table 8. Comparison of the responses between the predicted value from the model and the validation run using a HiScreen Capto MMC ImpRes column with the predicted settings

Identity	Aggregate at 90% yield (%)	Yield at 1% aggregate (%)	Pool volume (CV)
Predicted value	0.34	NA	4.1
HiScreen Capto MMC ImpRes	0.39	> 95	4.0

Conclusions

This work describes a rapid procedure to establish a robust second step in B/E mode for the purification of a MAb using Capto MMC ImpRes. The medium provides high yield of monomeric MAb, as well as good clearance of aggregate, HCP, and leached protein A. A model approach to the choice of running parameters defined by the desired purification performance was also shown.

Ordering information

Product	Code number
Capto MMC ImpRes, 1 L*	17-3716-03
HiScreen Capto MMC ImpRes, 1 × 4.7 mL	17-3716-20
Tricorn 5/20 column	28-4064-08

* Several pack sizes available. Visit www.gelifesciences.com for more information.

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