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High Throughput Chromatography Resin Screening and Purification Process Optimization of a Fab Antibody Fragment Using 96-Plates and PRC Prepacked Columns

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1. Summary

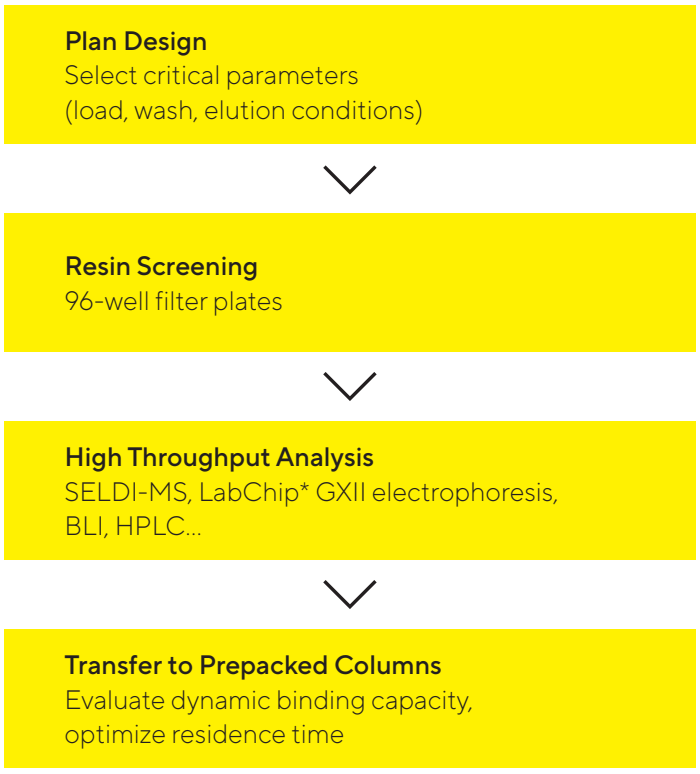
- This study describes the development of a three-step purification process for a fragment of antibody (Fab) expressed in Pichia pastoris.
- High-throughput chromatography resin screening was performed, based on 80 process conditions per day in 96-well filter plates with minimal sample consumption (<5 mL), and conditions transferred to column chromatography using 1 mL PRC prepacked columns.
- The combination of high throughput screening and fast analytics allowed the development of the capture step in four days and the transfer on lab-scale columns in four extra days. The screening of the intermediate and polishing steps were conducted in one day each as well as the transfer on lab-scale columns.
- The final three-step process, consisting of a cation exchange capture step in bind | elute mode followed by two negative steps (flow through mode) of respectively mixed-mode (intermediate step) and anion exchange (polishing step) chromatography resulted in an overall recovery of 70% of a 99% pure Fab fragment containing less than 30 ppm host cell proteins (HCP).

2. Introduction

Fab antibody fragments are increasingly used for therapeutic applications. Expressed at high yield, they are very specific and lack the Fc portion of the molecule that may lead to undesirable immunological reactions into patients.

In order to accelerate process development (i.e., the choice of appropriate resins and conditions to purify a Fab), 96-well filter plates were used. The processing of these plates includes "batch chromatography" steps, similar to any column chromatography experiment. Various chromatography modes are screened with minimal sample consumption. The use of plates allows easy handling by manual or robotic workstation-assisted operations. Purification conditions optimized in plates are then transferred and scaled to column chromatography using PRC prepacked columns or conventional equipment. The principle of the screening strategy is illustrated in Figure 1.

Figure 1
Screening Strategy Principle



3. Materials and Methods

3.1. Sample

The starting material was a clarified Pichia pastoris feedstock with a total protein concentration of ~2 mg/mL, and a Fab concentration of approximately 0.6 mg/mL. Conductivity and pH of the feedstock were 8 mS/cm and pH 6.0, respectively.

3.2. Design of Experiments and High Throughput Resin Screening

Capture, intermediate and polishing step strategies were screened on 9 different chromatography resins using 96-well filter plates. The screening was carried out in 1 mL 96 well filter plates filled manually with 50 µL of chromatography resin per well.

The plates were processed on a Freedom EVO* 150 robotic platform (Tecan): the storage solution from each well was removed using a Te-VacS vacuum manifold (Tecan) and a batch chromatography sequence was applied on the plates. Each step was carried out at room temperature (RT) under orbital shaking (1200 rpm) on a Te-shake agitator and fractions were collected by aspiration using the vacuum manifold.

For the capture step, 176 conditions were tested in bind | elute mode on nine different chromatography resins, including cation exchanger and mixed-mode resins (Table 1). Then, 13 conditions were screened in a negative mode on MEP HyperCel resin and on HyperCel STAR AX resin for the intermediate and polishing steps, respectively (Table 2). Process conditions applied for the screening of the capture step were based on a selection of 1 to 4 typical conditions for each type of resin chemistry. Both intermediate and polishing step conditions were determined using a full factorial design of experiments (DoE) software (Minitab*). Other softwares can be also used such as MODDE®.

* Freedom EVO is a registered trademark of Tecan Trading AG; Minitab is a registered trademark of Minitab LLC; LabChip is a registered trademark of Caliper Life Sciences Inc.

Table 1
Binding and Elution Conditions Tested on MEP, HEA and PPA HyperCel Mixed-Mode resins and CM Ceramic HyperD® F Cation Exchange Resin for Screening of the Capture Step

Resins	Equilibration | Washing	Elution 1	Elution 2	Elution 3	Elution 4
MEP, HEA, PPA HyperCel	20 mM Na Phosphate, pH 6.0	100 mM Na Acetate, pH 5.5	100 mM Na Acetate, pH 5.0	100 mM Na Acetate, pH 4.0	100 mM Na Acetate, pH 3.0
	20 mM Na Phosphate, 500 mM NaCl, pH 6.0				
	PBS, pH 7.4				
	20 mM Na Phosphate, 500 mM NaCl, pH 7.4				
	50 mM Tris/HCl, pH 8.0				
	50 mM Tris/HCl, 500 mM NaCl, pH 8.0				
CM Ceramic HyperD® F	50 mM Na Acetate, pH 4.5, +/- 150 mM NaCl	Equilibration buffer + 0.05 M NaCl or 50 mM Na Acetate, pH 5.5	Equilibration buffer + 0.1 M NaCl or 20 mM Na Phosphate pH 6.0	Equilibration buffer + 0.15 M NaCl or 50 mM Tris-HCl pH 7.0	Equilibration buffer + 0.3 M NaCl or 50 mM Tris-HCl pH 8.0
	50 mM Na Acetate, pH 5.0, +/- 150 mM NaCl				
	50 mM Na Acetate, pH 5.5 +/- 150 mM NaCl				

Table 2
Conditions of Screening in Negative Mode on MEP HyperCel Resin for the Intermediate Step and on HyperCel STAR AX Resin for the Polishing Step

Binding Conditions on MEP HyperCel Resin		Binding Conditions on HyperCel STAR AX Resin	
pH	NaCl [mM]	pH	NaCl [mM]
4.5	0	4.5	0
5.0	0	5.0	0
5.5	0	5.5	0
4.5	150	4.5	75
5.0	150	5.0	75
5.5	150	5.5	75
4.5	300	4.5	150
5.0	300	5.0	150
5.5	300	5.5	150

Strip elution conditions applied for all binding conditions were 100 mM Na acetate, pH 3.0 on MEP HyperCel resin and 50 mM Na acetate (pH 4.5, 5.0 or 5.5) + 1 M NaCl on HyperCel STAR AX resin

3.3. Transfer to Column Chromatography

The optimal conditions optimized in plates were transferred to lab-scale columns using 1 mL PRC prepacked columns filled with CM Ceramic HyperD® F, MEP HyperCel or HyperCel STAR AX resins. The residence time applied ranged between 3 and 5 minutes depending on the Resin type (150 to 60 cm/hr, respectively).

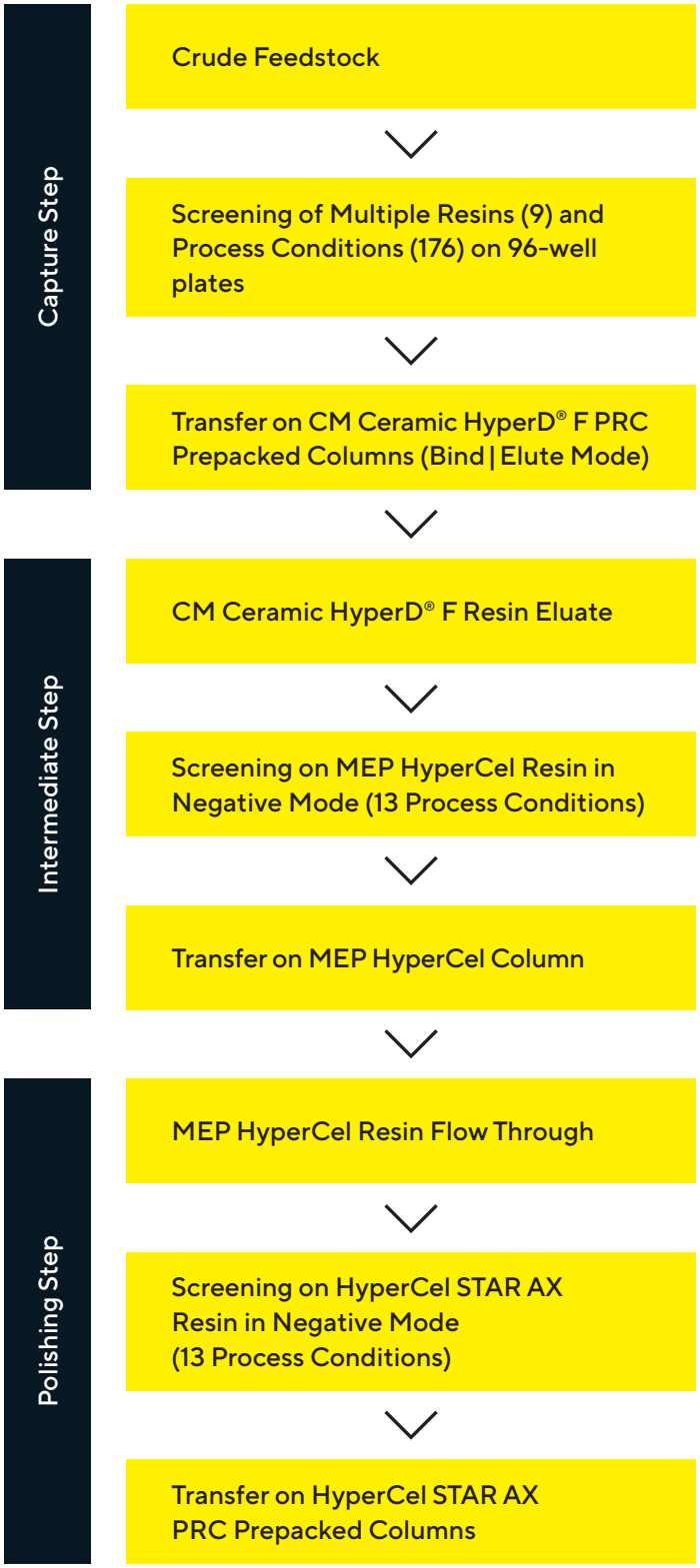
3.4. Transfer to Column Chromatography

- Total Protein Content was assessed using Bradford assay (Pierce).
- Chromatography fractions were analyzed using SELDI-MS (Bio-Rad), microfluidic electro phoresis on LabChip GXII system (Caliper LS | PerkinElmer) and | or SDS-PAGE (Invitrogen).
- HCP concentration was evaluated using anti-Pichia pastoris HCP ELISA test (Cygnus Technologies).

4. Results

The process development approach is described in Figure 2.

Figure 2
Process Development Approach Using 96-Well Plates and PRC Prepacked Columns



4.1. Development of the Fab Capture Step

4.1.1. Screening of Fab Capture Strategies in 96-Well Plate

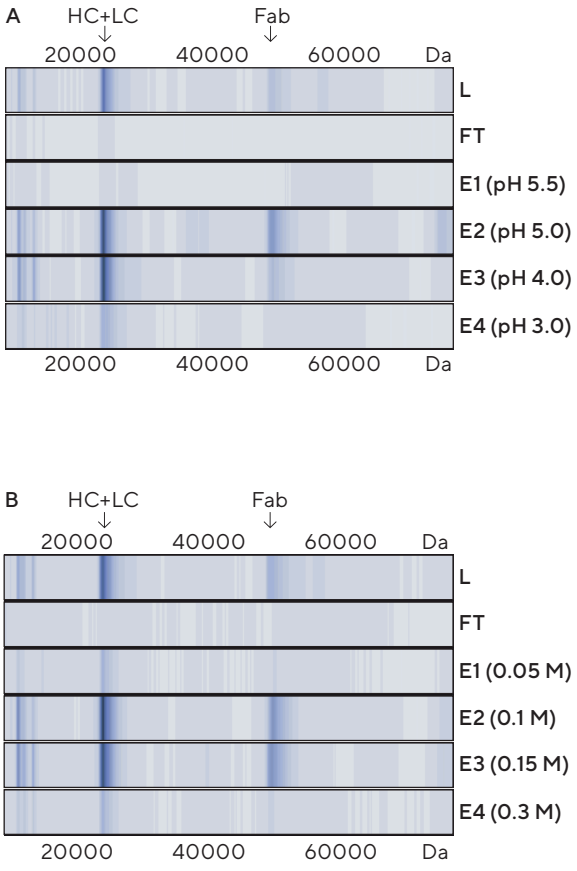
For the capture step, 176 conditions were tested in bind | elute mode on nine different chromatography resins, including cation exchange and mixed-mode resins, using 96-well filter plates.

The capture of the Fab appeared to be mostly effective on a mixed-mode resin (MEP HyperCel resin) or on a weak cation exchanger (CM Ceramic HyperD® F resin) (Figures 3A and 3B). In both cases, the Fab was retained on the resin (not detected in the flow through fraction) and mainly recovered at pH 5.0 from MEP HyperCel resin or in 0.10 to 0.15 M NaCl from CM Ceramic HyperD® F resin.

The proposed purification strategies resulting from the screening were either:

- **loading of the feedstock directly on MEP HyperCel resin at pH 6.0 and recovery of the Fab at pH 5.0, or**
- **loading of the feedstock on CM Ceramic HyperD® F resin after adjustment at pH 5.0 and recovery of the Fab at pH 5.0 + 0.15 M NaCl.**

Figure 3
SELDI-MS Profiles of the Fractions After Screening on **(A)** MEP HyperCel Resin or **(B)** CM Ceramic HyperD® F Resin in 96-Well Plate



(A) Equilibration in 20 mM Na phosphate, pH 6.0. L: Direct load of the sample; FT: Flow through; Elution in 100 mM Na acetate E1: pH 5.5; E2: pH 5.0; E3: pH 4.0; E4: pH 3.0 or **(B)** Equilibration in 50 mM Na acetate, pH 5.0. L: Load of the sample after adjustment at pH 5.0; FT: Flow through; Elution in 50 mM Na acetate pH 5.0 + E1: 0.05 M; E2: 0.1 M; E3: 0.15 M; E4: 0.3 M NaCl HC: heavy chain; LC: light chain; Fab: fragment of antibody

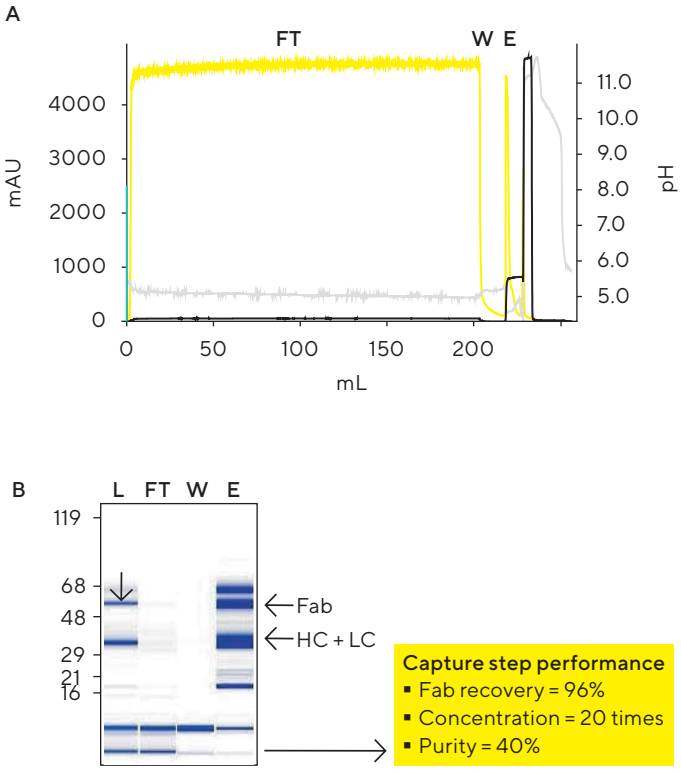
4.1.2 Transfer of the Fab Capture Step and Evaluation of the Dynamic Binding Capacity (DBC) on CM Ceramic HyperD® F and MEP HyperCel Resins

Based on the 96-well plate screening, the capture step was transferred on CM Ceramic HyperD® F and MEP HyperCel PRC prepacked columns (Figures 4 and 5).

It confirmed the capture of the Fab on the two selected resins. Elution steps at different salt concentrations on the cation exchange column evidenced that 0.3 M NaCl was preferable to 0.15 M to maximize Fab recovery (data not shown), allowing for a final recovery of 96% at 0.3 M NaCl (Figure 4).

It also confirmed that the major contaminants (free light and heavy chains) were binding on the two resins and were either co-eluted with the Fab on the cation exchanger, or partly separated from the Fab on the mixed-mode resin between pH 5.2 (Fab) and 4.0 (LC + HC). Besides this, the DBC of the two resins was evaluated at lab-scale, showing that the DBC on the cation exchanger (60 mg/mL) was greater than the mixed-mode resin in the conditions tested. The relatively low DBC of MEP HyperCel resin for the Fab (2.4 mg/mL) was due to the strong binding of free LC and HC that competed with the Fab. Based on the capacity and recovery yield at elution, **cation exchange chromatography was selected over mixed-mode chromatography as the capture strategy of the Fab.**

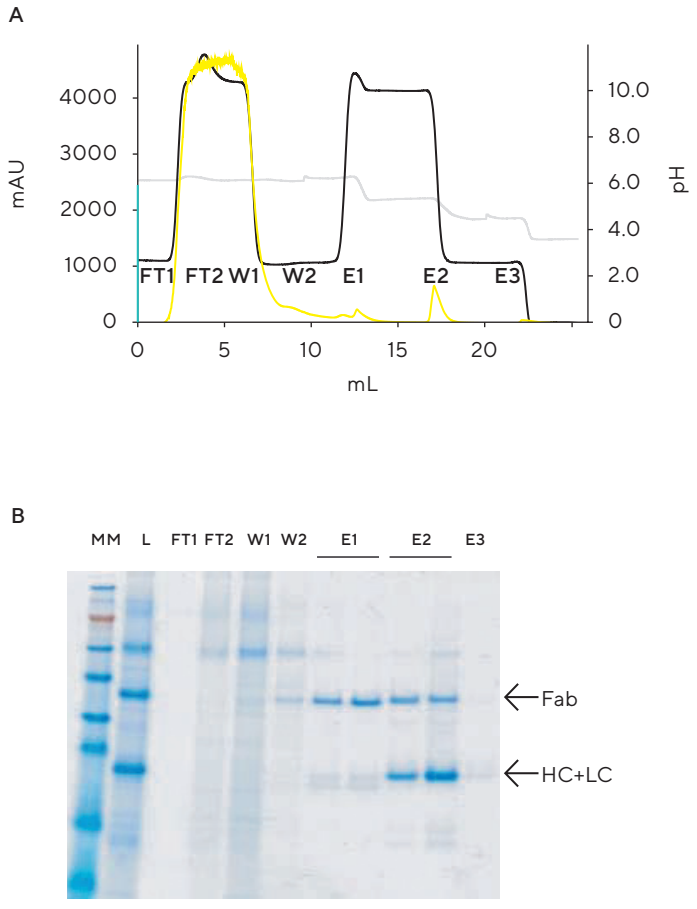
Figure 4
Capture Step on CM Ceramic HyperD® F (Cation Exchanger) PRC Prepacked Column: Chromatogram **(A)** and LabChip Analysis of Fractions **(B)**



L: Load; FT: Flow through; W: Wash; E: Elution. **(A)** Chromatography run: Column: 1 mL PRC prepacked column; Equilibration and washing: 50 mM Na acetate, pH 5.0; Loading: 200 mL of 2-fold diluted feedstock adjusted at pH 5.0; Elution: equilibration buffer + 0.3 M NaCl; Flow rate: 150 cm/hr, 3 minutes residence time. **(B)** Analysis of fractions using LabChip GXII electrophoresis HC: heavy chain; LC: light chain; Fab: fragment of antibody

The development of the capture step on MEP HyperCel evidenced that the main contaminants (free LC and HC) could be partly separated from the Fab on MEP HyperCel resin as they elute later (pH 4.0) than the Fab (4.0 to 5.2), suggesting that free chains had more affinity for MEP HyperCel resin than the Fab (Figure 5). Therefore MEP HyperCel resin was considered for the intermediate step, in a negative mode, to remove these major contaminants (section 3.2).

Figure 5
Capture Step on MEP HyperCel (Mixed-Mode) PRC Prepacked Column: Chromatogram **(A)** and SDS-PAGE Analysis of Fractions **(B)**



L: Load; FT: Flow through; W: Wash; E: Elution; MM: Molecular weight marker. **(A)** Chromatography run: Column: 1 mL PRC prepacked column; Equilibration and washing: 20 mM Na phosphate, pH 6.0; Loading: 8 mL of 2-fold diluted feedstock in equilibration buffer; Elution: E1 pH 5.2; E2 pH 4.0; E1 pH 3.0; Flow rate: 60 cm/hr, 5 minutes residence time. **(B)** Analysis of fractions using SDS-PAGE. HC: heavy chain; LC: light chain; Fab: fragment of antibody

4.2. Intermediate Purification Step Using Mixed-Mode Chromatography on MEP HyperCel Resin

4.2.1. Screening of Conditions on 96-well Filter Plate

Process conditions were screened in 96-well ScreenExpert plate in negative mode, on the eluate of the cation exchange column, following a DoE approach that includes three pHs (pH 4.5, 5.0 and 5.5) combined with 3 NaCl concentrations (no salt, 150 mM, 300 mM). Fab purity and recovery in the flow through fraction after MEP HyperCel resin chromatography are strongly dependent on pH and salt concentration (Figures 6A and 6B) and a compromise had to be found between the two parameters. Indeed, a decrease of the pH and of the ionic strength favored Fab recovery but lowered Fab purity. Figure 7 highlights the region of pH and conductivity where the optimal compromise of purity and recovery were reached in 96-well plate.

Figure 6
Fab Purity **(A)** and Recovery **(B)** in the Flow Through Fraction After MEP HyperCel Resin Negative Mode Batch Chromatography (Second Purification Step) as a Function of the Binding pH and Salt Concentration

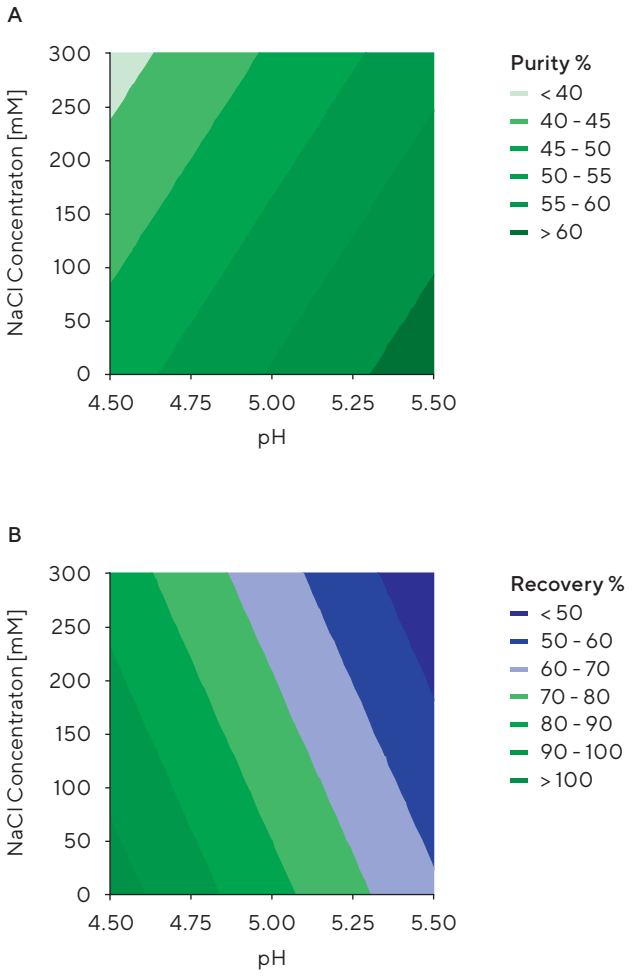
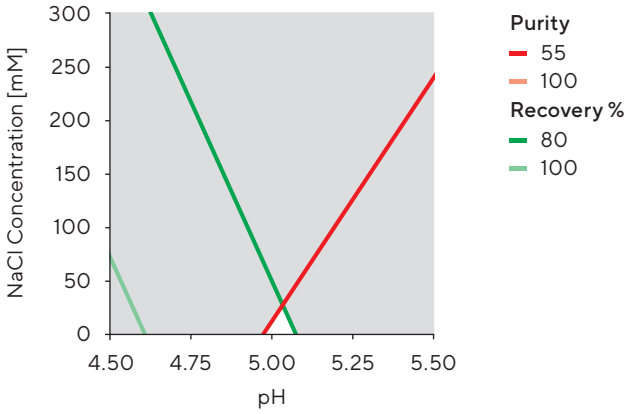


Figure 7
Optimization Range of Fab Purity and Recovery in the Flow Through Fraction After MEP HyperCel Negative Mode Batch Chromatography (Second Purification Step) as a Function of the Binding pH and Salt Concentration



Based on a DoE approach using 96-well plates, optimal Fab purity and recovery in negative mode was observed at a binding pH of 5.0 to 5.2 without addition of salt on MEP HyperCel resin.

4.2.2. Transfer of Optimized Conditions on MEP HyperCel Resin Mixed-Mode Column Chromatography

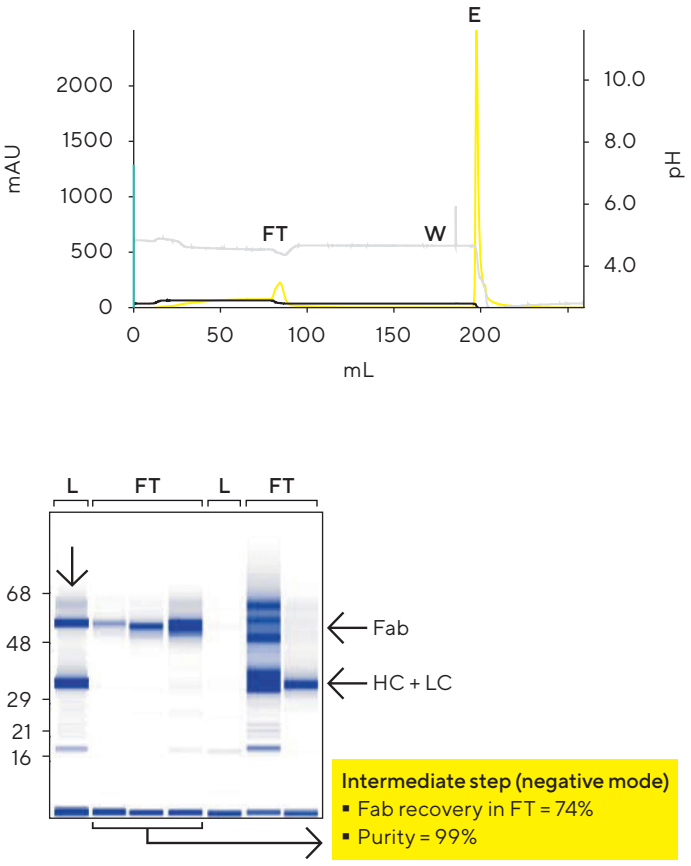
Transfer of the optimal conditions from the 96-well plate to PRC prepacked columns at pH 5.0, 5.1 and 5.2 showed that the optimal purity of the Fab was reached at pH 5.2, where recovery remained acceptable (i.e., ≥70%) (Table 3).
The loading condition selected for the intermediate purification step on MEP HyperCel resin was therefore pH 5.2.

Table 3
Intermediate Purification Step on MEP HyperCel PRC Prepacked Columns in Negative Mode at pH 5.0, 5.1 and 5.2

Equilibration pH	Fab Purity [%]	Fab Recovery [%]
5.0	71	100
5.1	89	86
5.2	99	71

Besides this, DBC of MEP HyperCel resin in negative mode at pH 5.2 was such that 0.8 mL of the first column eluate diluted 10-fold to reduce NaCl concentration from 0.3 to 0.03 M could be loaded per mL of MEP HyperCel resin without having free light and heavy chains in the flow through fraction (data not shown). Therefore a lab-scale column was packed with 10 mL of MEP HyperCel resin for the intermediate purification step of 60 mL of CM Ceramic HyperD® F resin column eluate diluted 10-fold (Figure 8). Analysis of the fractions confirmed that free chains were retained on the resin and eliminated from the Fab fraction. The recovery of the Fab in the flow through was 74% and the purity reached, 99% based on LabChip GXII analysis (Figure 8).

Figure 8
Intermediate Step: Transfer of Conditions on MEP HyperCel PRC Prepacked Column



L: Load; FT: Flow through; W: Wash; E: Elution
Column: 10 mL MEP HyperCel resin; Equilibration and washing: 50 mM Na acetate, pH 5.2; Loading: 60 mL of 10-fold diluted CM Ceramic HyperD® F eluate at pH 5.2; Elution: 50 mM Na acetate, pH 3.0; Flow rate: 150 cm/hr, 5 minutes residence time.

Conditions optimized in plates were successfully transferred. The Fab was recovered in the column flow through (FT) at pH 5.2, a significant increase of the Fab purity was observed, and most of the impurities including free light and heavy chains were eluted during the acidic strip elution, i.e., pH 3.0 (E).

4.3. Polishing Step by Anion Exchange Chromatography

4.3.1. Screening of Conditions on 96-Well Plates

A polishing step to decrease further HCP content in the recovered Fab fraction was screened in 96-well plate filled with HyperCel STAR AX resin, on the MEP HyperCel column eluate. ELISA anti-HCP assay of flow through fractions evidenced that the binding conditions yielding to a HCP content below 30 ppm were pH 4.5, 0 to 150 mM NaCl or pH 5.0 without salt (highlighted in Table 4), whereas recovery of Fab was satisfying in all the conditions tested. The contour plot of the HCP content as a function of pH and NaCl concentration confirmed that the conditions of the polishing step should range between pH 4.5 and 5.0, with 0 to 140 mM NaCl (Figure 9).

Table 4

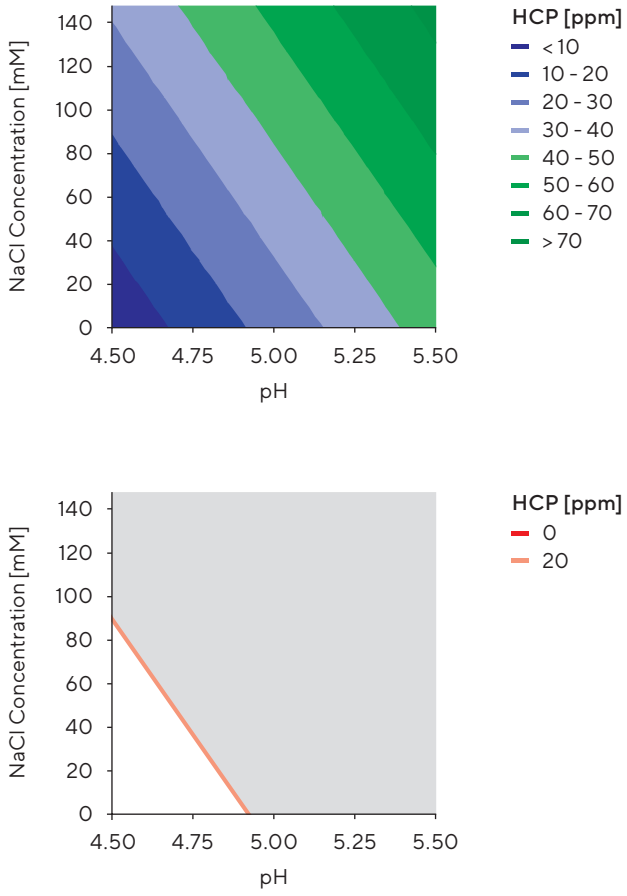
Fab Recovery and HCP Content for the 13 Screening Conditions Tested on HyperCel STAR AX Resin

Fractions	Fab Recovery in FT [%]	HCP [ppm][1]
Load	-	87
FT pH 4.5	**100**	**13**
FT pH 4.5 + 75 mM NaCl	**100**	**27**
FT pH 4.5 + 150 mM NaCl	**100**	**19**
FT pH 5.0	**100**	**23**
FT pH 5.0 + 75 mM NaCl [2]	96	32
FT pH 5.0 + 150 mM NaCl	100	65
FT pH 5.5	100	51
FT pH 5.5 + 75 mM NaCl	100	44
FT pH 5.5 + 150 mM NaCl	100	90

[1] ng/mg Fab
[2] Average values for the five replicates repeated at pH 5.0 + 75 mM NaCl in the frame of a full factorial DoE approach (face centered)

Figure 9

HCP Removal on HyperCel STAR AX Resin



HCP content in the flow through fraction after batch chromatography on HyperCel STAR AX resin in negative mode as a function of the binding pH and salt concentration

The screening showed that the Fab was recovered in the column flow through and that the HCP content was below 30 ppm when the binding was comprised between pH 4.5 and 5.0 and 0 to 140 mM NaCl.

4.3.2. Transfer of Optimized Conditions on HyperCel STAR AX Resin Column Chromatography

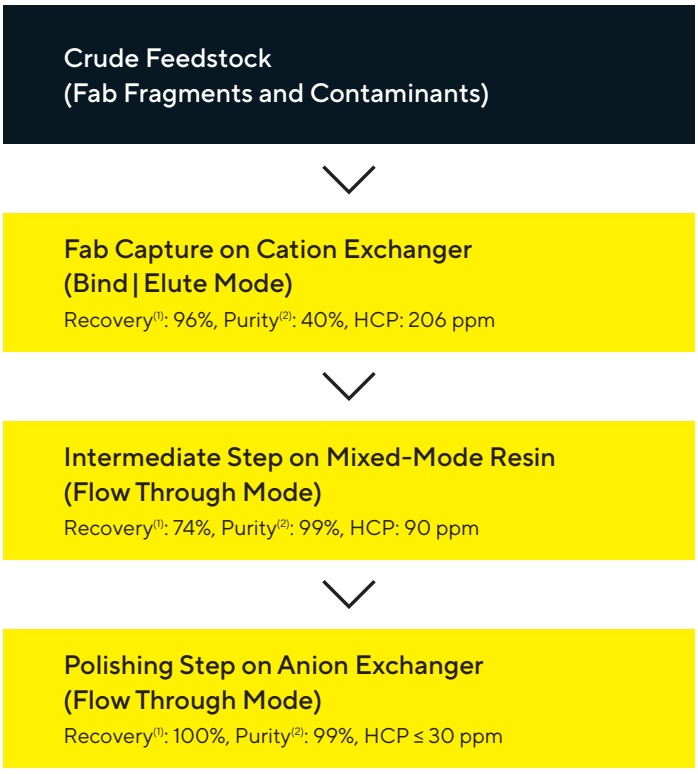
To confirm the conditions determined during the screening, a transfer of the polishing step on a HyperCel STAR AX PRC prepacked column was conducted at pH 4.5 and 5.0. HCP content in the flow through fraction was 15 and 30 ppm, respectively. The data obtained on column were in good agreement with the data generated on 96-well plates. **At the end of the three-step process, the Fab purity was estimated around 99% with not more than 30 ppm HCP. The overall process recovery was around 70%.**

5. Discussion and Conclusion

This example shows the benefits of the combination of high throughput screening using 96-well filter plates and transfer of the process steps in prepacked columns for the fast development of a three-step purification strategy of a Fab fragment (Figure 10).

- 80 chromatography conditions were screened and analyzed per day, significantly reducing process development duration
- Conditions optimized in plates were checked and confirmed by performing column chromatography runs
- Cation exchange capture on CM Ceramic HyperD® F resin acted as a first concentration (about 20-fold) step in the process, with medium purity (40%)
- Used in flow through mode, mixed-mode chromatography on MEP HyperCel resin was a powerful intermediate step to remove various contaminants, especially free light and heavy chains (Fab purity 99%)
- Anion exchange chromatography on HyperCel STAR AX final polishing step allows to decrease the contaminant level (HCP ≤30 ppm)

Figure 10
Overall Purification Scheme



(1) Recoveryevaluated using a Bradford assay
(2) Purity evaluated using the LabChip GXII system

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