High-throughput screening and process development for capture of recombinant pro-insulin from *E. coli*

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GE Healthcare
Life Sciences

Application note 28-9966-22 AA

Process development

High-throughput screening and process development for capture of recombinant pro-insulin from *E. coli*

This Application note describes a complete process development workflow from PreDictor™ plate screening to a 400 mL pilot-scale purification for capturing recombinant pro-insulin expressed in *E. coli*. PreDictor 96-well prefilled filter plates, Assist software and Tricorn™ 5/50 columns packed with 1 mL media (resins) were used to develop optimized conditions for the capture step. High-throughput screening quickly narrowed down the number of candidate chromatography media and suggested binding and elution conditions for further investigation. When capture step conditions were further optimized and verified, the purification was scaled up 400-fold. Scale-up was smooth and predictable, reaching a final-scale yield of 96% and a target molecule purity of 82%. Screening in PreDictor plates also saved considerable time and sample compared with column experiments. A second study (1) describes a similar workflow for the intermediate purification of the insulin obtained following the enzymatic cleavage of pro-insulin.

Introduction

Introducing high-throughput methods into process development workflows improves efficiency. Reduction in the time and amount of sample required to develop the chromatographic steps of a downstream purification process are examples of the gains that can be made. Today, GE Healthcare Life Sciences offers a wide range of products to improve the efficiency of process development.

PreDictor 96-well filter plates (prefilled with BioProcess™ chromatographic media) are designed for efficient, high-throughput initial screening of chromatographic conditions and media. Assist software simplifies the process of setting up experiments as well as data management and evaluation.

Defined conditions from the screening can then be verified and optimized with HiTrap™ or HiScreen™ prepacked chromatography columns. ÄKTA™ avant 25 chromatography system supports these efforts with both buffer preparation and DoE (Design of Experiments), which simplify optimization in the column format and promote scaling up the process to pilot level via, for example, HiScale™ 16/40 columns. Further scale-up is possible with ÄKTA avant 150 system and AxiChrom™ columns.

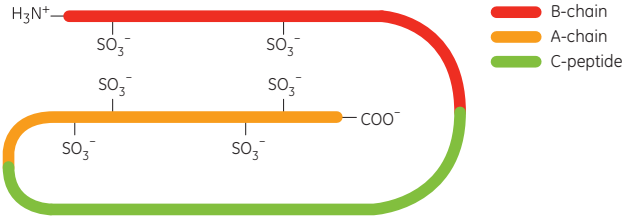


Fig 1. Schematic illustration of the pro-insulin molecule.

This Application note describes the use of GE Healthcare's process development platform to develop a robust capture method for recombinant pro-insulin (Fig 1). PreDictor plates and Assist software were used to determine a chromatographic medium for capture and identify promising binding and elution conditions (2). Based on the screening results, Capto™ MMC (a multimodal cation exchanger) was selected as the most promising medium due to its ability to bind sample without prior dilution. With the binding and elution conditions found in the screening experiments as starting point, the capture step was optimized on a Tricorn 5/50 column packed with 1 mL of medium. Once a robust capture protocol had been established, the process was successfully scaled up from Tricorn to HiScreen prepacked columns, HiScale 16/40 column (20 cm bed height) packed with Capto MMC, and finally to an AxiChrom 50/300 column (19.5 cm bed height, 400 ml packed bed volume).

Materials and methods

Screening with PreDictor plates

Whenever possible, experiments with PreDictor plates were performed with fully automated protocols on a Tecan™ Freedom EVO™-2 200 Robotic System. More complex protocols such as sample handling were carried out manually. Liquid removal was performed by vacuum or centrifugation throughout the study.

The pro-insulin used in all experiments originated from *E. coli*. It was supplied by BIOMM S.A., Belo Horizonte, Brazil. The pro-insulin solution was subjected to sullfitolysis to hinder the formation of disulfide bridges. The suspension that contained 8 M urea was approximately 10 mg/mL in recombinant pro-insulin and 18 mg/mL in total protein. The conductivity of the sample was approximately 14 mS/cm. PreDictor experiments followed the illustration shown in Figure 2. The detailed experimental procedure can be found in the reference 3. Conditions studied are presented in Results and discussion.

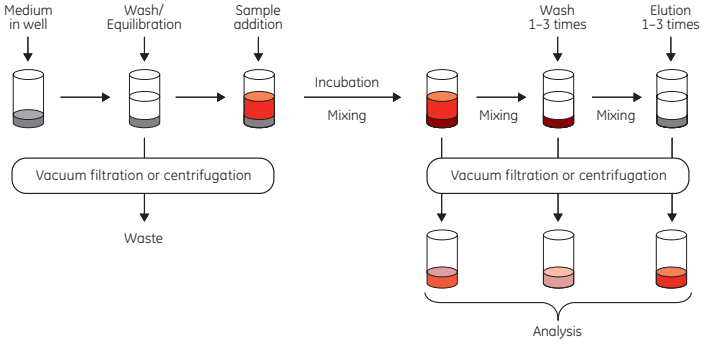


Fig 2. Schematic illustration of the workflow of a batch experiment in the wells of a PreDictor plate. The same steps would be employed in a column experiment, that is, equilibration, sample addition, wash, and elution. The gray color in the wells is chromatography medium; red shades (red and pink) describe different concentrations of protein solution. Brown is medium with bound sample.

Analysis

In the PreDictor binding studies, capacities were measured from analyses of the flowthrough fraction. In the elution studies, the first elution fraction was evaluated. Start samples were analyzed in all studies. All analyses were performed by anion exchange chromatography on a Mono Q™ 5/50 GL column (Fig 3).

Column: Mono Q 5/50 GL (1 mL)
Sample: 25 µL sample injected using flushed injection
Buffer A: 100 mM Tris-HCl, 1 mM Na EDTA, pH 2.5 in 8 M urea
Buffer B: Buffer A + 1 M NaCl
Flow rate: 1 mL/min
Elution: Step 1, 0% B for 4 CV;
Step 2, 16% B for 3 CV;
Step 3, 26% B for 3 CV, linear gradient to 50% B for 3 CV;
Step 4, 100% B for 4 CV, 0% B for 4 CV
System: ÄKTAmicro™
Detection: UV-1 monitor, 280 nm

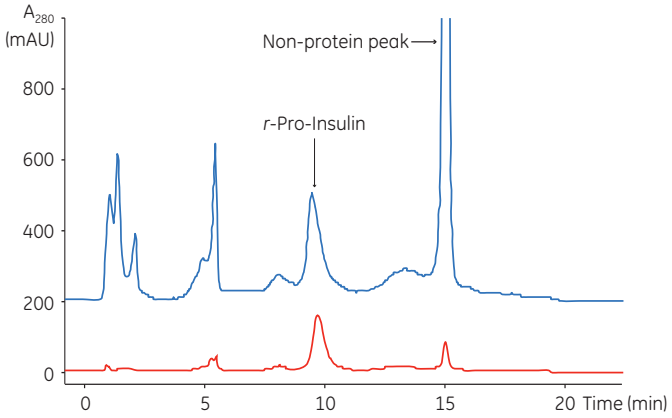


Fig 3. Overlay analysis chromatograms of the crude sample (blue curve) and an elution fraction (red curve).

The pro-insulin sample concentration was determined by integrating the area of the peak eluting at a retention time of 9 to 10 min and relating its surface area to that in the crude sample:

$$Concentration_{sample} = Concentration_{crude\ sample} \times Peak\ area_{sample}/Peak\ area_{crude\ sample}$$

The resulting pro-insulin concentration in the flowthrough or first elution fraction for each condition was used as in-data in Assist software where the response surfaces for experimental evaluation were generated.

Column experiments

Column experiments comprising optimization, dynamic binding capacity experiments, a robustness study, and scale-up, were performed with the Capto MMC multimodal medium on chromatography systems suitable for the column dimensions. Table 1 summarizes the columns, systems, samples, and purification conditions for these experiments.

Table 1. Summary of the Capto MMC column experiments in process development

Study	Column	V_c (mL)	System	Binding buffer*	Sample load (mL)	Flow rate (mL/min)
Dynamic binding capacity	Tricorn 5/50	1	ÄKTAmicro	A+150 mM NaCl	10	0.2
Elution optimization	Tricorn 5/50	1	ÄKTA avant 25	A+150 mM NaCl	2.5	0.2
Robustness study	Tricorn 5/50	1	ÄKTA avant 25	A+150 mM NaCl	2.5	0.2
Scale-up	2 × HiScreen 4.7/10	9.4	ÄKTA avant 25	A+150 mM NaCl	24	1.9
	HiScale 16/40	40	ÄKTA avant 150	A+150 mM NaCl	100	8
	AxiChrom 50/300	400	ÄKTA avant 150	A+150 mM NaCl	960	80

* Buffer A: 100 mM Tris-HCl, 1 mM Na EDTA, pH 2.5 in 8 M urea

All eluent buffers were prepared in 8 M urea and all experiments were concluded with 1 M NaOH cleaning-in-place (CIP) followed by storage in 20% ethanol. Detection was performed at 280, 405, and 260 nm. In the preliminar elution experiments salt/pH gradients were used while optimization, robustness and scale-up experiments were performed as step elutions. As in the PreDictor experiments, sample analyses were performed by the previously described Mono Q method.

Results and discussion

Screening experiments for binding

Binding experiments were performed on a selection of ion exchange and multimodal media; the PreDictor plates contained 2 µL or 6 µL of SP Sepharose™ Fast Flow, Capto S, Capto MMC (2). The small media volumes (2 and 6 µL) enabled binding experiments by overloading the media with the buffered sample (200 µl solution 2.5 mg/ml in respect to pro-insulin per well) contained in 8 M urea without consuming more than 15 mL of crude sample for the binding study. The binding with respect to both the initial salt concentration and the pH value of the binding buffer was examined. Table 2 summarizes the test conditions for each medium.

Table 2. Summary of media and parameters in the binding experiments conducted on PreDictor plates

Experiment	Factor 1 pH	Factor 2 NaCl (mM)
Binding study – CIEX screening plate Capto S, 2 µL Capto MMC, 6 µL SP Sepharose Fast Flow, 6 µL	3.4–5.0	0–300
Binding study – Capto MMC, 6 µL	3.0–7.0	0–300

In all binding experiments, the flow-through fraction was collected and analyzed with respect to non-bound pro-insulin as compared to the start sample, which gives an indication of the binding capacity at each condition. The resulting response surfaces for all media, generated using Assist software, are shown in Figure 4.

Capto S and SP Sepharose Fast Flow indicate high binding capacities at the lowest pH tested (i.e. 3.4) and no salt. Capto MMC binds at 150 mM salt and higher pH compared to these two media. Since the starting sample of the fusion protein has an ionic strength close to 150 mM NaCl, high binding capacity at this concentration is an advantage.

A second binding study was thus performed with Capto MMC and a broader parameter interval intended to reveal the optimum binding for this medium. As the resulting Figure 5 shows, the highest binding capacities (red/orange zone) for pro-insulin binding to Capto MMC are obtained at pH 5 (or just above) and 0 to 160 mM NaCl. It was decided to continue with Capto MMC and to study conditions for elution.

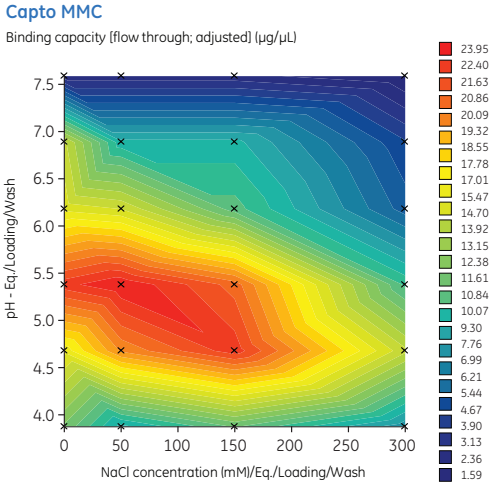


Fig 5. Response surface for binding of pro-insulin on Capto MMC as a function of NaCl concentration (0 to 300 mM) and buffer pH (4 to 7.5). Assist software was used in visualizing this data.

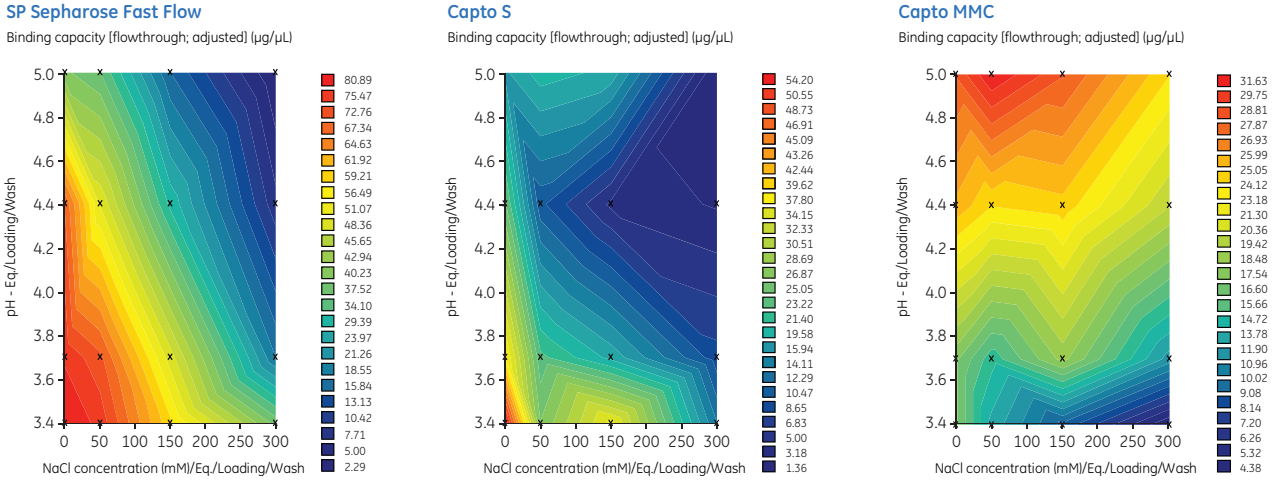


Fig 4. Response surfaces generated by Assist software for pro-insulin binding as a function of NaCl concentration (x-axis) and buffer pH (y-axis) for SP Sepharose Fast Flow, Capto S, and Capto MMC, respectively. The range of binding capacities achieved is shown to the right of each surface. Black crosses represent actual data between results that have been interpolated.

Screening experiments for elution

PreDictor plates with 50 µL Capto MMC media volume were used for elution studies. This ensured sufficient loading to detect the target molecule without overloading the medium. The amount of protein applied in the loading step corresponded to 70% of the binding capacity that was estimated in the binding study i.e., 180 µl sample, 5 mg/ml in respect to pro-insulin. The elution study was performed using a range of eluent compositions; pH 3.7 to 7.6 and 150 to 1000 mM NaCl. The evaluation procedure was the same as for the binding study, but now the first elution fraction was analyzed. This showed the conditions required to obtain elution in the column verification work that followed.

As only the first elution fraction was analyzed, one may not expect full yield in this step. The highest yield achieved was 70% and was found at pH 7.5 and a NaCl concentration above 600 mM (Fig 6.)

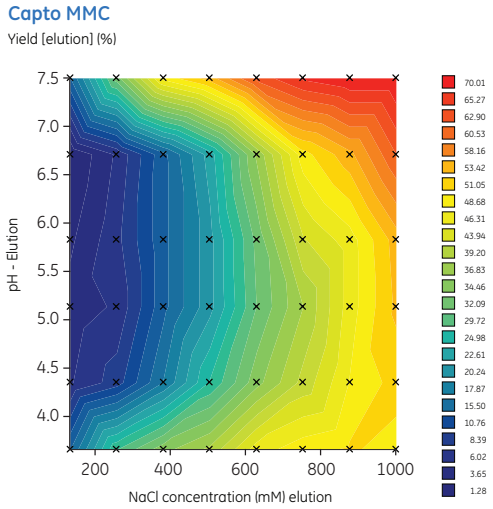


Fig 6. Elution of pro-insulin (first elution fraction) on Capto MMC as a function of NaCl concentration (150 to 1000 mM) and buffer pH (4 to 7.5). Assist software was used in obtaining these data.

Optimization in Tricorn columns on ÄKTA avant 25

The high-throughput screening experiments on PreDictor plates suggested that the best conditions for pro-insulin capture would be binding at around pH 5 and a NaCl concentration of 50 to 150 mM on Capto MMC followed by eluting at a pH greater than 7 and a NaCl concentration above 600 mM.

With these parameters added as factors in a DoE protocol, the capture step was optimized on Capto MMC packed in a Tricorn column (1 mL bed volume). As 150 mM NaCl corresponds to the isotonic salt concentration found in the start sample, this salt concentration was an obvious starting point for binding since it eliminated the need to dilute sample prior to loading. Binding buffer pH was set at 5.2 and the pH of the start sample was set accordingly. In the first column experiment, elution with a salt gradient was tested by loading 20 mg pro-insulin (2 mL sample), 50 mM sodium acetate buffer, pH 5.2 in 8 M urea on the 1 mL column and eluting with a linear salt gradient of 150 to 1000 mM NaCl for 7 column volumes (CV).

Figure 7 shows the results. The fraction collected at the maximum height of the elution peak was analyzed on Mono Q and the resulting chromatogram compared to that of the crude sample and one flowthrough fraction.

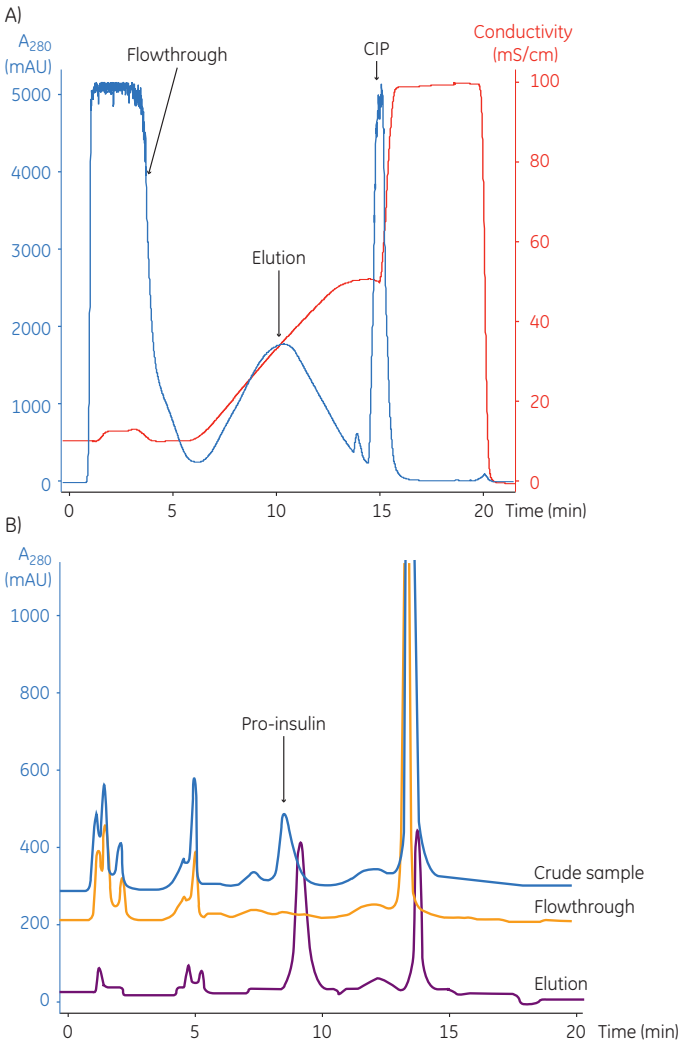


Fig 7. A) A 2 mL crude sample, pH 5.2 in 8 M urea, loaded on a Tricorn 1 mL 5/50 column packed with Capto MMC column and eluted by a linear salt gradient from 150 to 1000 mM NaCl for 7 CV. B) Corresponding Mono Q analysis of crude sample, flowthrough (FT), and one elution fraction (collected at the main elution peak maximum). In both A) and B), detection was at 280 nm.

Analysis of the flowthrough fraction (Fig 6B) showed good binding of the target molecule with no pro-insulin detected in the flowthrough. The nonprotein impurity seemed to be low binding as it appeared in the flowthrough fraction while the corresponding peak in the elution fraction was significantly smaller. This indicated good capture and purification of pro-insulin.

However, a large peak was seen during CIP (Fig 7A) suggesting that high salt concentration alone was not adequate to recover all of the pro-insulin. Experience with several other target proteins indicates that multimodal media frequently require more than just high ionic strength for efficient elution (4).

Figure 8 shows the capture and analysis results where the salt gradient was supplemented with a pH 5.2 to 7.5 gradient.

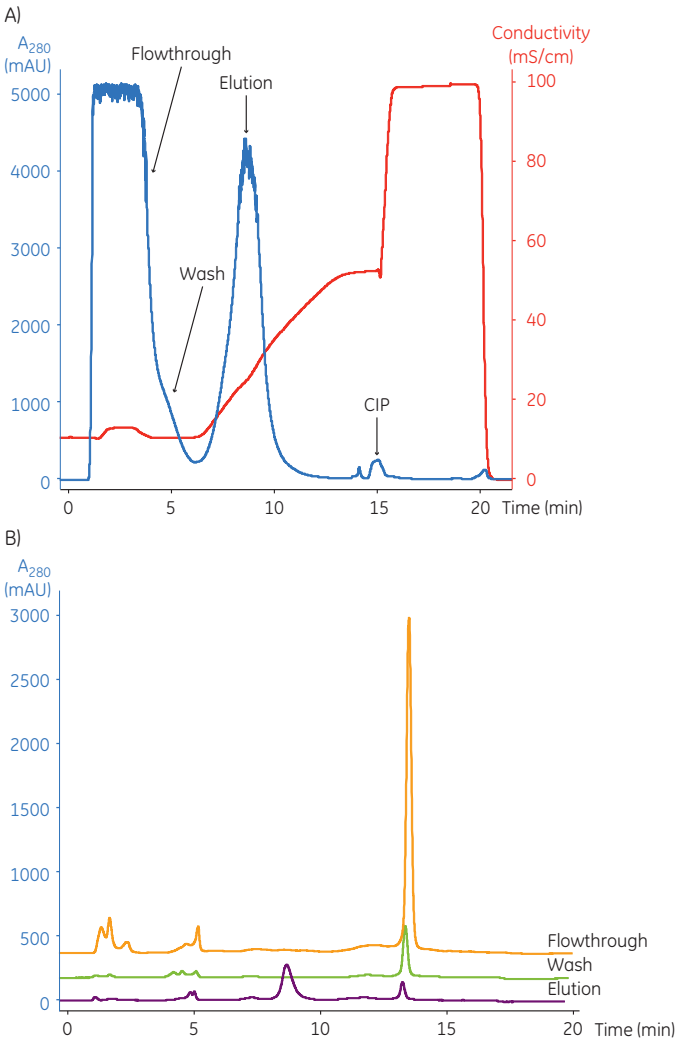


Fig 8. A) A 2 mL crude sample, pH 5.2 in 8 M urea, loaded on a Tricorn 1 mL column packed with Capto MMC and eluted with a linear combined salt and pH gradient from 150 to 1000 mM NaCl and pH 5.2 to 7.5 for 7 CV. B) Corresponding Mono Q analysis of the flowthrough, wash, and pooled fractions in the main elution peak. In both A) and B), detection was at 280 nm.

Comparing chromatograms for the constant pH (Fig 7A) and the pH gradient (Fig 8A) capture experiments revealed that a combined pH and salt gradient gave both a narrow elution peak and a high yield, neither of which was achieved when salt gradient elution alone was employed.

Dynamic binding capacity experiments

Once promising conditions for binding and eluting pro-insulin had been established, attention was turned to dynamic binding capacity (DBC). This was determined by overloading the column with crude sample and collecting and analyzing fractions to determine the point at which pro-insulin breakthrough occured.

Based on DBC experiments, the loading in the experimental work was set to 25 mg pro-insulin (2.5 mL crude sample, approx. 80% DBC) to secure complete binding.

Elution optimization

Aiming at a step elution mode, elution conditions were optimized using the buffer prep and DoE tools of ÄKTA avant 25. A full factorial design with three center points based on two variables (pH and NaCl concentration) each at three levels was set up to determine the salt concentration and the pH needed to obtain sufficient purity and yield (above 80% and 95% respectively). The area of the pro-insulin peak as well as the area percent of pro-insulin in the analysis chromatogram (purity) were set as responses. See Table 3 for details.

Table 3. Design variables, values for the elution optimization and purity data of pro-insulin in the eluted peak

Run	NaCl (mM)	Elution pH read†	Area (mAU × ml)	Purity (%)
1*	450	7.1	196	76
2	150	7.1	181	79
3*	450	7.1	196	81
4	150	8	246	83
5*	450	7.1	187	82
6	750	8	251	84
7	750	7.1	241	82
8	450	6.2	69	71
9	450	8	250	84
10	150	6.2	37	53
11	750	6.2	116	78

* Center points

† 8 M urea influences the pH reading, settings in ÄKTA avant was approximately 1 pH-unit lower

Figure 9 shows the pro-insulin peak area in the collected elution peak as a function of pH and NaCl concentration. This clearly demonstrates that the optimal elution for pro-insulin is found at high pH, whereas an increase in the concentration of NaCl above 150 mM has only a minor effect. The purities achieved were also highest at high pH. It was decided to perform the elution at pH 8 and 150 mM NaCl.

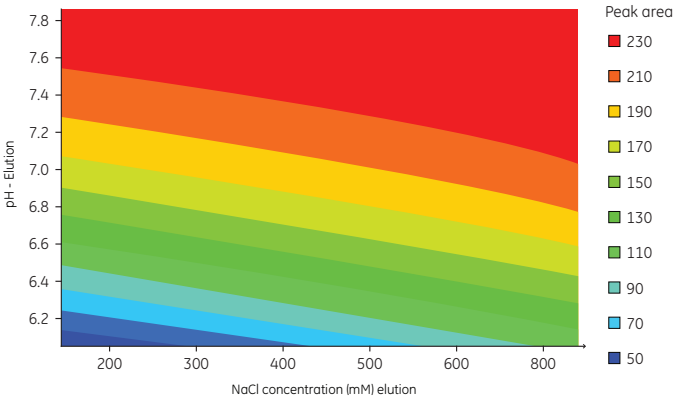


Fig 9. Response surface for the peak area of pro-insulin as a function of pH and NaCl concentration in mM. $R^2 = 0.989$, $Q^2 = 0.736$. ÄKTA avant was used in obtaining these data.

Robustness study

To conclude process development, a robustness study was performed on 1 mL Tricorn 5/50 columns packed with Capto MMC using the optimized elution conditions of pH 8 and 150 mM NaCl. The robustness study was designed using a Placket Burman DoE (5) based on four variables (two chromatography media batches, two crude sample batches, elution pH 7.8 to 8.2 and load volume 2.3 to 2.7 mL) with 150 mM NaCl in all eluent buffers. Figure 10 shows the scaled and centered coefficients for the purity data (all above 80% purity) from the eluted peaks as function of the variable parameters. It is clear that no significant model terms can be detected. The yield was approximately 95% for all conditions in this study.

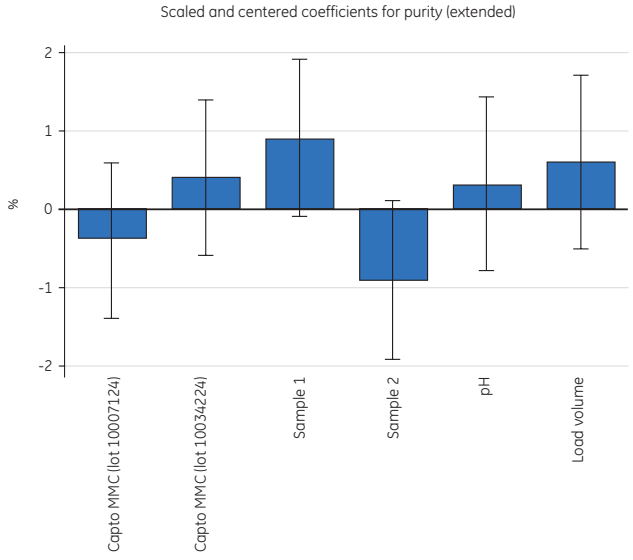


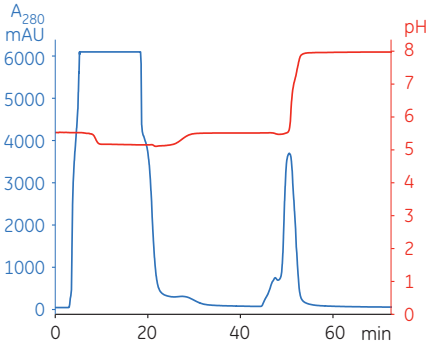
Fig 10. Scaled and centered coefficients for purity as a function of four variable parameters; medium lot (×2), sample (×2), pH, and load volume.

Scale-up experiments

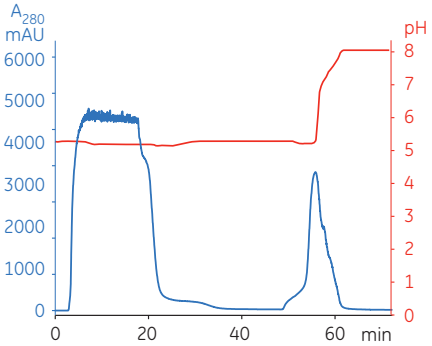
Columns with 20 cm bed heights were used for 9, 40, and 400-fold scale-up by increasing column diameter while keeping other parameters such as residence time and sample load/mL media constant. Other conditions were similar to those found in the optimization study on the Tricorn 5/50 column packed with Capto MMC (loading at pH 5.2 and elution at pH 8, both in the presence of 150 mM NaCl).

Two HiScreen Capto MMC columns were connected in series to give 20 cm bed height. In addition, a HiScale 16/40 column (diameter 16 mm) and an AxiChrom 50/300 (diameter 50 mm) were packed with Capto MMC to bed heights of 20 and 19.5 cm, respectively. The capture experiment was performed at 240 cm/h at all three extended scales (5 min residence time). Fractions from the flowthrough and the eluted peaks were analyzed on the Mono Q column. Results and purity data (Fig 11 and Table 4) show that the capture step of the pro-insulin purification was successfully transferred from the 1 mL Tricorn 5/50 column to the 400 mL AxiChrom 50 column. The resulting pro-insulin purity was 82% and the yield 96% measured at the 400 mL scale.

A) HiScreen Capto MMC (two columns connected in series, bed height 20 cm), ÄKTA avant 25 system



B) Capto MMC packed in HiScale 16/40 (bed height 20 cm), ÄKTA avant 150 system



C) Capto MMC packed in AxiChrom 50/300 (bed height 19.5 cm), ÄKTA avant 150 system

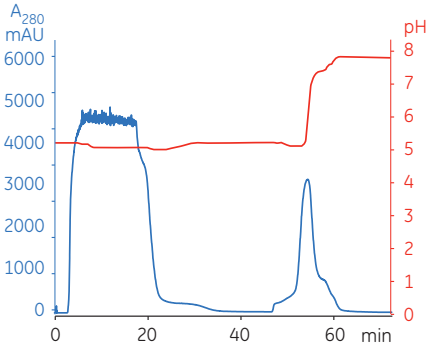


Fig 11. Chromatograms from loading pro-insulin crude sample on Capto MMC at pH 5.2 and 150 mM NaCl followed by step elution to pH 8 on A) HiScreen (9 mL), B) HiScale (40 mL) and C) AxiChrom (400 mL) columns.

Table 4. Scale-up and purity data. All columns were loaded with 25 mg pro-insulin/mL medium

Column*	Scale-up factor	Crude sample load (mL)	Pro-insulin purity (%)
Tricorn 5/50	1	2.5	83
HiScreen Capto MMC × 2	9.4	23.5	86
HiScale 16/40	40	100	84
AxiChrom 50/300	400	960	82

* Total packed bed heights were 20 cm, except for AxiChrom 50, which was 19.5 cm

After completing the process development, the amount of sample used to perform the presented work as well as the number of days of laboratory work was estimated (Table 5).

Table 5. Sample consumption and time for one person to for the complete process development

	Time	Amount of sample[†]
Screening in PreDictor plates	7 days	300 mg
Column experiments (optimization, robustness and scale-up)	10 days	11 g

[†] 1 mL of crude sample contained 10 mg of the pro-insulin target molecule

Conclusions

High-throughput screening with PreDictor plates and the Assist software allowed quick selection of most suitable chromatography medium and identification of promising binding and elution conditions for the capture of recombinant pro-insulin expressed in *E. coli*. This gave a fast and confident start to the purification process development.

Based on these screening experiments, Capto MMC was the medium of choice for further work due to its ability to bind sample without prior dilution. The capture step was further optimized in a Tricorn 1 mL column packed with Capto MMC, again based on the binding and elution conditions determined by the screening experiments. Once the optimized protocol had been confirmed to be robust, the process was successfully scaled up from a 1 mL Tricorn 5/50 column to a 400 mL AxiChrom 50/300 column. The resulting purity for the capture step was 82% with a yield of 96%.

The overall outcome demonstrates the value of introducing high-throughput methods into process development workflows. In this PreDictor plate screening example, media and condition selection was completed in one week using 30 mL of crude sample (300 mg of the target molecule). When UV absorbance in a plate reader is sufficient for evaluation, media screening can be finalized within two days.

The screening described here enabled fast development of a pilot-scale process (400 mL AxiChrom column) within 4 weeks.

References

1. Application note: High-throughput screening, process development, and scale-up of an intermediate purification step for recombinant insulin. GE Healthcare 29-0018-56.
2. Application note: *High throughput process development with PreDictor plates*, GE Healthcare, 28-9403-58.
3. Instruction: *PreDictor plates*, 28-9258-34.
4. Application note: *Optimizing elution conditions on Capto MMC using Design of Experiments*. GE Healthcare 11-0035-48.
5. Method Validation in Pharmaceutical Analysis. A Guide to Best Practices. Joachim Ermer and John H. McB. Miller (Eds), p 149-150, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim (2005).

Ordering information

Related literature

Data files	Code no.
PreDictor 96-well filter plates and Assist software	28-9258-39
AxiChrom columns - Simple operation & Superior performance	28-9441-99
ÄKTA avant	28-9573-45
HiScreen prepacked columns	28-9305-81
Tricorn Empty - high performance columns	18-1147-36
Capto MMC	11-0035-45

Handbooks	Code no.
Ion Exchange Chromatography & Chromatofocusing, Principles and Methods	11-0004-21
High-throughput process development with PreDictor plates	28-9403-58

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