High-throughput screening of HIC media in PreDictor plates for capturing recombinant Green Fluorescent Protein from *E. coli*

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Process development

High-throughput screening of HIC media in PreDictor™ plates for capturing recombinant Green Fluorescent Protein from *E. coli*

Hydrophobic interaction chromatography (HIC) is a powerful purification technique where the type and density of the ligand, pH and salt of binding conditions, temperature and the nature of the target protein are highly significant parameters when determining selectivity. This study demonstrates a rapid, high-throughput process development workflow using parallel screening in 96-well plates to determine the most suitable HIC media and conditions for capturing recombinant Green Fluorescent Protein expressed in *E. coli*. Chromatography media were ranked and their elution profiles predicted in a fast and effective way, saving both time and sample compared with traditional column screening. Results showed that binding and elution conditions could be fine-tuned to obtain high protein purity.

Introduction

The hydrophobic ligand, ligand density, target protein, mobile phase, type of salt and salt concentration all play significant roles in determining the final selectivity and capacity of HIC media. Unlike other chromatographic techniques where 'standard proteins' may provide useful guidelines for predicting chromatographic performance, many parameters must be determined and optimized by experiments for HIC-based purifications.

Furthermore, experience shows that the results are often hard to predict, even when the properties of the target protein are well known (1). High-throughput process development in plates is thus a valuable tool for rapid parallel screening of HIC media and conditions.

Hydrophobic interaction chromatography media from the GE Healthcare BioProcess™ media family are available in a series of PreDictor 96-well filter plates that comprise both single-medium and multi-media screening formats.

This Application note describes a high-throughput screening study using multi-media plates. It demonstrates a process development workflow that determines the most promising HIC media and shows how salt type and concentration conditions may be tailored to successfully capture recombinant Green Fluorescent Protein (rGFP) expressed in *E. coli*. The results are compared with those obtained using traditional packed-bed chromatography.

The work involved the following main steps:

1. Solubility test for rGFP to establish the 'salt stability window' for three salt types and find the concentration at which precipitation occurs, i.e. to find maximum salt concentrations for binding.
2. Screening media and conditions (salt type and concentration variations) in PreDictor HIC screening plates.
3. Screening selectivity with different binding concentrations of the selected salt type, also in PreDictor HIC screening plates.
4. Comparing the screening results with those obtained with small-scale columns to check the correlation between the two formats and to further study the suitability of the candidate media for their allocated purification task. Dynamic binding capacity in columns was also determined. The sample consumed and the time taken for the plate screening was compared to that required for the small-scale column experiments.

Materials and methods

Sample preparation

rGFP, a stable protein of 238 amino acids with a molecular weight of 28 kDa, pI 6.2 and specific absorbance at 490 nm, was recovered from homogenized and frozen *E. coli*. After thawing, the homogenate was pH-adjusted, centrifuged, filtered through a 0.45 µm filter, and buffer exchanged before its concentration and start purity value were determined.

Solubility study

Prior to screening, the solubility of rGFP was measured by light scattering at 350 nm in the presence of three salt types (sodium chloride, sodium sulfate and ammonium sulfate) at different concentrations and pH 7 in 96-well UV collection plates.

PreDictor HIC screening plates

The series of HIC PreDictor plates available from GE Healthcare includes two screening plates each containing four HIC media. The low hydrophobicity range plate comprises the following media, in approximate order of increasing hydrophobicity:

- Butyl S Sepharose™ 6 Fast Flow
- Octyl Sepharose 4 Fast Flow
- Butyl Sepharose 4 Fast Flow
- Capto™ Octyl

The high range hydrophobicity plate comprises:

- Phenyl Sepharose 6 Fast Flow (low sub)
- Capto Butyl
- Phenyl Sepharose 6 Fast Flow (high sub)
- Capto Phenyl (high sub)

Both plates were used in this study. Media volumes in all cases were 50 µL, i.e. the volume recommended for elution studies (2).

PreDictor plate experimental procedure

Batch uptake experiments on PreDictor plates were executed according to the instructions for PreDictor plates and with the help of Assist software. The experimental steps were as follows:

Equilibration 1 to 3:	200 µL equilibration buffer, 1 min incubation
Sample loading:	200 µL clarified rGFP sample (approx. 1 mg/mL), 60 min incubation
Wash 1 to 3:	200 µL equilibration buffer, 1 min incubation
Elution 1 to 3:	200 µL different elution buffers, 1 min incubation
Strip:	200 µl strip buffer, 1 min incubation

The sample loaded corresponds to a non-overloaded amount for most media, i.e. conditions recommended for elution studies (2). Equilibration buffer and clarified rGFP sample contained the salt type or its start salt concentration intended for the two screening experiments (see Figs 1 and 2).

All steps included mixing on a microplate shaker at 1100 rpm. Collected solutions were removed by centrifugation and all fractions were collected in UV-readable plates. rGFP concentration in the fractions was determined from absorbance measurements at 490 nm. Purity was determined by comparing the ratio:

$$Purity = A_{490}/(A_{280}-A_{310})$$

Absorbance at 310 nm is measured to determine the contribution from light scattering (Rayleigh scattering) and is subtracted from the absorbance at 280 nm as the A_{310} signal contributes to the A_{280} signal, but not the A_{490}.

The purification factor was calculated as the ratio 'purity in the eluate/purity in the crude sample' as shown below:

$$Purification\ factor = \frac{Purity\ in\ eluate}{Purity\ in\ crude\ sample}$$

Experimental plate layout

The experimental conditions used during screening of media and conditions (varying salt type and concentrations) and of binding (salt) conditions are shown in Figures 1 and 2 respectively as plate designs in Assist software.

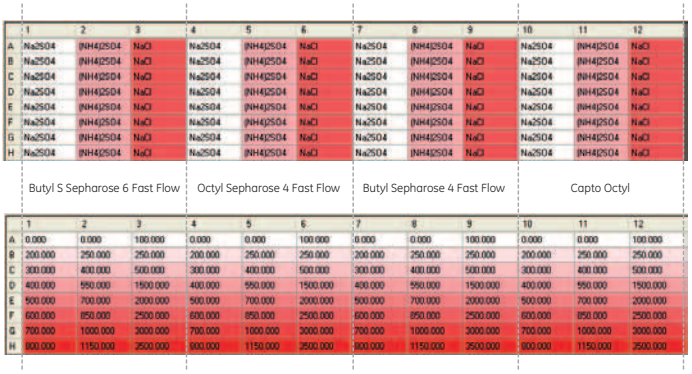

Fig 1. Plate design for screening media and salt type viewed for the low hydrophobicity screening plate. The same distribution of factors was applied for the high hydrophobicity screening plates. Salt concentrations in the elution step increase from row A to H as seen in the lower plate lay-out.

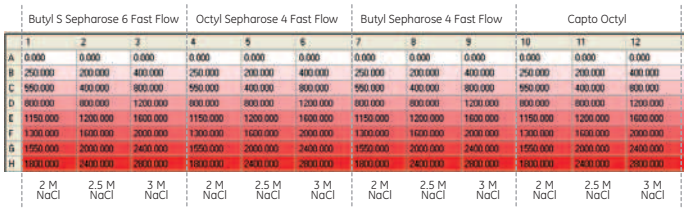

Fig 2. Plate design for screening binding conditions viewed for the low hydrophobicity screening plate. The same distribution of factors was applied for the high hydrophobicity screening plates. The concentrations (NaCl) during equilibration and loading are listed below each column. Salt concentrations in the elution step increase from row A to H.

Column experiments

Table 1 lists the steps and running conditions for the traditional packed-bed chromatography column experiments against which the PreDictor plate results are compared. All runs were made using ÄKTA™ avant 25 chromatography system on Tricorn™ 5/50 columns packed with 1 mL of the appropriate HIC medium. rGFP concentration was approx. 1 mg/mL.

Table 1. Summary of running conditions for the packed bed column experiments

Step	Col. vol. (CV)	Flow (mL/min)	Description
Equilibration	5	1	Start buffers:
			50 mM phosphate, pH 7, 4 M NaCl
			50 mM phosphate, pH 7, 3 M NaCl
			50 mM phosphate, pH 7, 2.5 M NaCl
			50 mM phosphate, pH 7, 2 M NaCl
Sample load	2.5 or 9	1	2.5 mL (for selectivity studies)
			9 mL (for capacity studies)
Wash 1	4	1	One of the start buffers for the specific run
Elution	10 + 8	1	Elution buffer: 50 mM phosphate, pH 7
			Linear gradient: 0-100% for 10 CV
			Gradient delay: 100% for 8 CV
Wash 2	5	0.5	Strip buffer: 30% isopropanol
Re-equilibration	5	1	Buffer: 50 mM phosphate, pH 7

Results

Solubility study

The 'salt stability window' for rGFP in clarified cell culture supernatant showed that the protein was stable in all tested concentrations of sodium chloride and sodium sulfate. For ammonium sulfate, solubility started to decrease just before salt concentration reached 1.5 M. Figure 3 summarizes the solubility results. To avoid precipitation and maximize binding (1), the following salt concentrations in the binding buffer (50 mM phosphate at pH 7) were selected; 1 M Na_2SO_4, 1.2 M $(NH_4)_2SO_4$, 4 M NaCl.

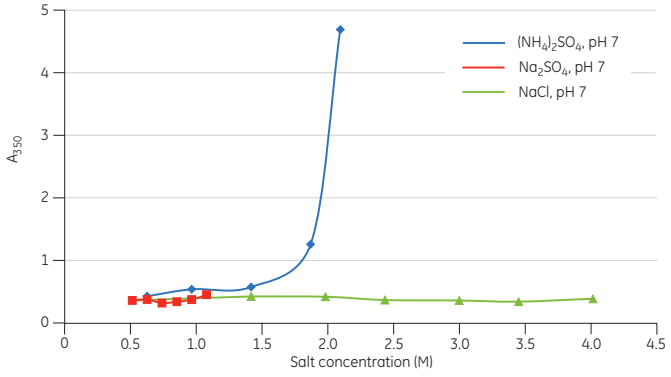

Fig 3. Solubility of rGFP in clarified cell culture supernatant at pH 7 with three different salt types (sodium chloride, sodium sulfate and ammonium sulfate).

Screening media and salt type

rGFP is a relatively hydrophobic protein and is expected to be more hydrophobic than many *E. coli* host cell proteins (HCP). An elution study with varied elution conditions may thus reveal conditions where more target protein is eluted than HCPs. In a batch experiment, the purification factor achieved at different elution conditions may indicate selectivity between target protein and impurities. In cases where the purity is higher at low salt concentrations, we would expect to see a chromatogram where the impurities elute before the target protein. If, on the other hand, the purification factor is independent of the elution conditions used, we may expect chromatographic results where rGFP and the impurities co-elute as one peak. Figure 4 shows a guide to interpreting such data.

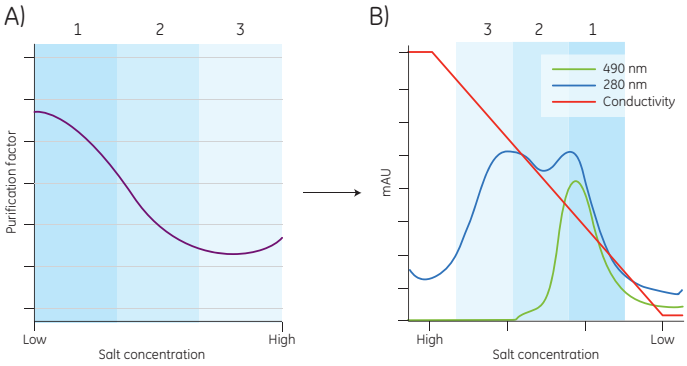

Fig 4. Comparison of batch and column data. A) purification factor as a function of salt concentration in elution, B) the corresponding chromatogram. Note that chromatographic elution on a HIC medium starts at a high salt concentration. The chromatogram has been divided in three parts. Corresponding salt concentrations for the batch experiments are indicated in A.

Figures 5 and 6 show yield and purity results from the elution fractions obtained from the screening of the eight PreDictor HIC media. Yield measurements (Fig 5) show low concentrations of rGFP in the elution fractions from Capto Butyl, Phenyl Sepharose 6 Fast Flow (high sub) and Capto Phenyl (high sub).

No rGFP was found in the flowthrough for these latter three media, indicating that the hydrophobic interaction of rGFP is too strong under the conditions tested (these media are considered to be the most hydrophobic of those investigated). Even though harsher conditions might be able to elute rGFP, all three media were thus excluded from further evaluation.

In related results, Butyl S Sepharose 6 Fast Flow reveals high levels of rGFP in the collection plate from the flowthrough directly after sample loading. This indicates low capacity for the target protein. Butyl S Sepharose 6 Fast Flow is thus not a candidate for the capture step. It was, however, kept in the screening study as it revealed an interesting purification factor pattern (Fig 6).

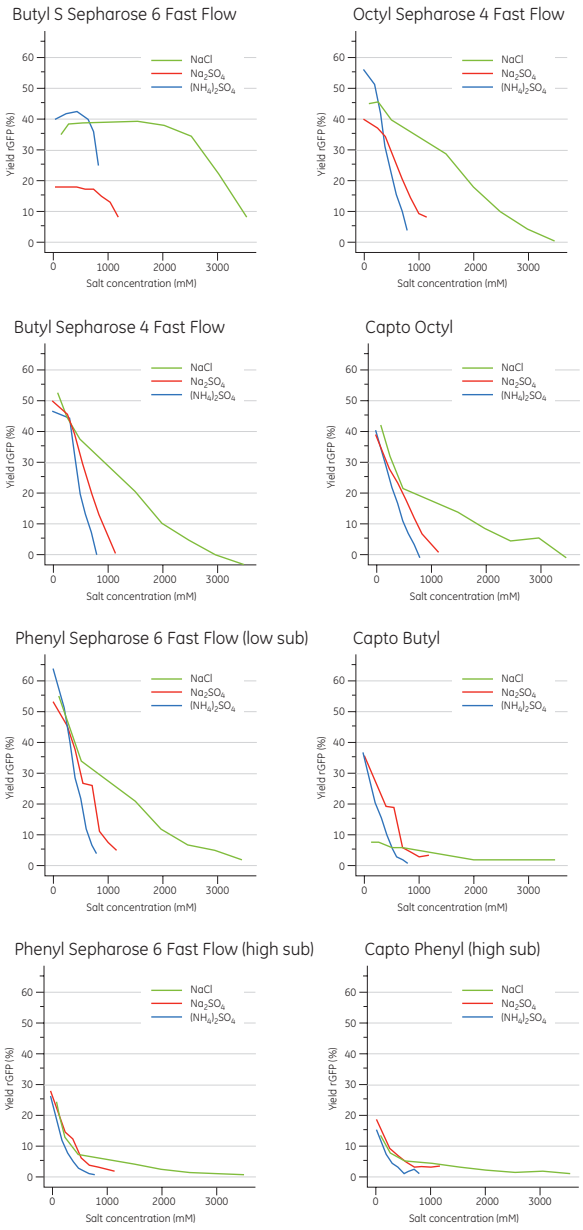

Fig 5. rGFP yield data (from elution fractions) of the HIC media screening to identify the media and salt type with best selectivity for rGFP. Low yield is seen for Capto Butyl, Phenyl Sepharose 6 Fast Flow (high sub), and Capto Phenyl (high sub) indicating that the hydrophobic interaction of rGFP is too strong under the conditions tested

Figure 6 shows purity data from screening suitable media (the four most interesting plus Butyl S Sepharose 6 Fast Flow) and salt types. The purification factor shows the same picture for the four most interesting media (Octyl Sepharose 4 Fast Flow, Butyl Sepharose 4 Fast Flow, Capto Octyl and Phenyl Sepharose 6 Fast Flow [low sub]); all show a decreasing purification factor with increasing salt concentration. We can expect that HCPs will tend to elute prior to rGFP, which will elute in the latter part of the gradient. This suggests the possibility of eluting impurities while still binding rGFP.

In some circumstances, the purity result may resemble a 'U-shape', depending on the salt concentration. However, high purity at high salt concentrations may possibly be due to an error in the calculated ratio caused by too small amounts being eluted.

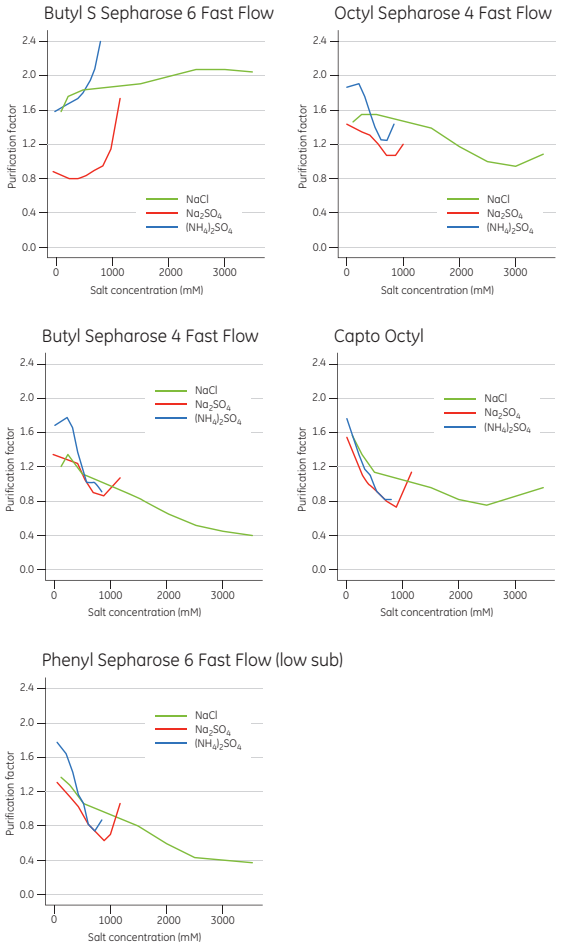

Fig 6. rGFP purity data from elution fractions.

The purity data for Butyl S Sepharose 6 Fast Flow and NaCl as salt type show a high purification factor independent of salt concentration. This value even declines slightly at decreasing NaCl elution conditions (seen as a falling line). This suggests that this medium will bind fewer HCPs than rGFP, and that rGFP and impurities will co-elute in one peak in the upper part of the chromatographic gradient.

These interpretations of the batch experiments are verified by column experiments for three of the media. All three salts gave approximately the same purification profile. Therefore, only one of them (NaCl) was chosen for column verification. Figure 7 shows the chromatograms.

As expected from the plate experiments, Butyl S Sepharose 6 Fast Flow loaded with 4 M NaCl in the buffer revealed one peak perfectly overlapping with the impurity peak eluting early in the chromatogram. In the case of Octyl Sepharose 4 Fast Flow and Phenyl Sepharose 6 Fast Flow (low sub), the chromatograms showed rGFP eluting at the end of the gradient and a significant amount of the impurities eluting before. We may thus conclude that the chromatographic results, i.e. peak patterns, correlate well with the purity revealed in the plate experiments. Furthermore, the results indicate that rGFP may still bind at salt concentrations even lower than the 4 M NaCl used here.

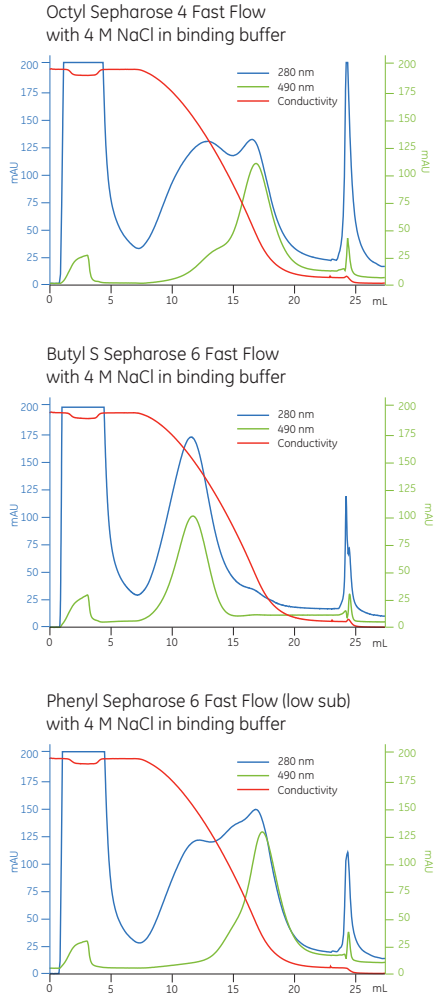

Fig 7. Chromatograms of the Tricorn 5/50 runs. Columns were packed with 1 mL medium. The peak in the flowthrough is rGFP-related impurities and the sharp peak at the end of the chromatogram is strongly-bound components that eluted in 30% isopropanol.

Screening binding conditions with NaCl

As the initial screening revealed a possibility to remove impurities in the flowthrough fraction by using a lower salt concentration for binding, a second screening was performed, again using HIC PreDictor plates. The aim was to screen for the lowest salt concentration in the start buffer that maintains binding capacity for rGFP and minimizes the risk of co-eluting HCPs, thus achieving high purity. For the sake of simplicity, only one salt type (NaCl) was investigated.

NaCl was tested in the binding buffer at three concentrations (2 M, 2.5 M and 3 M). Again, the elution fractions from the 96-well plate were evaluated to provide selectivity data. This was facilitated by determining the purification factor in each fraction.

The four most promising HIC media were evaluated; Octyl Sepharose 4 Fast Flow, Butyl Sepharose 4 Fast Flow, Capto Octyl and Phenyl Sepharose 6 Fast Flow (low sub). For all but Octyl Sepharose 4 Fast Flow, rGFP was completely bound at all salt concentrations studied. However for Octyl Sepharose 4 Fast Flow at 2 M NaCl, a small but significant amount of rGFP was found in the flowthrough fraction, indicating weak binding. Elution data for 2 M NaCl and Octyl Sepharose 4 Fast Flow were not evaluated further. In general, and as expected when looking at HIC elution salt concentrations, all four media showed enhanced yield as salt concentration decreased in the elution step (data are not shown but the results are similar to Fig 5).

The purification pattern obtained in batch format is, as indicated earlier, expected to correspond to a certain chromatographic peak pattern. Figure 8 summarizes purity data. It can be seen that Octyl Sepharose 4 Fast Flow displays rather constant purity at 2.5 M NaCl while at 3 M NaCl, low purity is observed at higher salt concentrations.

Butyl Sepharose 4 Fast Flow, Capto Octyl and Phenyl Sepharose 6 Fast Flow (low sub) all show similar purity profiles. More HCP relative to rGFP will elute at higher salt concentration (early in a chromatogram), whereas rGFP will elute later in the gradient as salt concentration decreases.

However, Capto Octyl binds rGFP most strongly of these four media, and its yield of rGFP in elution fractions was also significantly lower compared to the other three (data not shown). The low yield indicates difficulties in eluting rGFP from this medium. It will probably elute as a very broad peak or even be eluted in the CIP fraction.

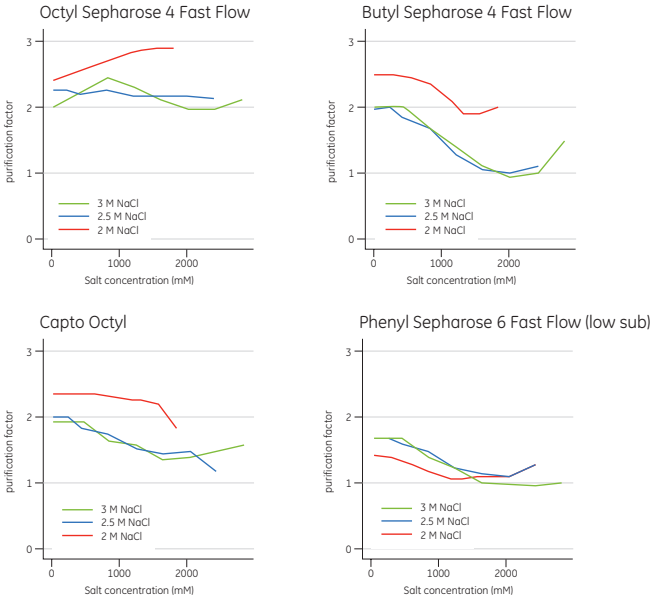

Fig 8. rGFP purity data (from elution fractions) from HIC media screening to identify a suitable initial salt concentration that gives best selectivity for rGFP.

Traditional chromatography column experiments with Tricorn 5/50 columns packed with 1 mL of each of the remaining media binding at 2 M NaCl were then performed. As a comparison, these columns were also run with 2.5 M and 3 M NaCl in the binding buffer. If 2 M NaCl is sufficient for achieving a satisfactory result, this will reduce the total amount of NaCl used at process scale.

The results (Fig 9) verify that rGFP is difficult to elute from Capto Octyl. It elutes late in the gradient and with the conditions used, some of the protein also elutes in the CIP step. Examining the chromatograms from Butyl Sepharose 4 Fast Flow clearly shows that fewer HCPs elute in the gradient part of the chromatogram when loading at 2 M NaCl compared to 2.5 and 3 M NaCl. In other words, less HPC's bind to the column when loading at 2 M NaCl. This is in agreement with the PreDictor results where binding at 2 M NaCl and eluting with the lowest salt concentrations revealed the highest purification factors (see Fig 8).

Results for Phenyl Sepharose 6 Fast Flow (low sub) are more difficult to interpret, possibly because the yield achieved in the first elution fraction in the batch experiments is slightly lower compared to Butyl Sepharose 4 Fast Flow. This is seen in the chromatogram as a broader rGFP peak for Phenyl Sepharose 6 Fast Flow (low sub). The chromatographic results for binding at 2 M NaCl also reveal that Phenyl Sepharose 6 Fast Flow (low sub) shows good purity. Thus, both this medium and Butyl Sepharose 4 Fast Flow should be chosen for further optimization in column format.

Dynamic binding capacity

Finally, the dynamic binding capacities (DBC) at 10% breakthrough at 1 min residence time for rGFP in clarified cell culture supernatant were determined by frontal analysis for the two most promising media; Butyl Sepharose 4 Fast Flow and Phenyl Sepharose 6 Fast Flow (low sub). To confirm the low capacities found in batch experiments for Octyl Sepharose 4 Fast Flow with 2 M NaCl for binding, this condition was also included in the DBC study. Table 2 shows the results.

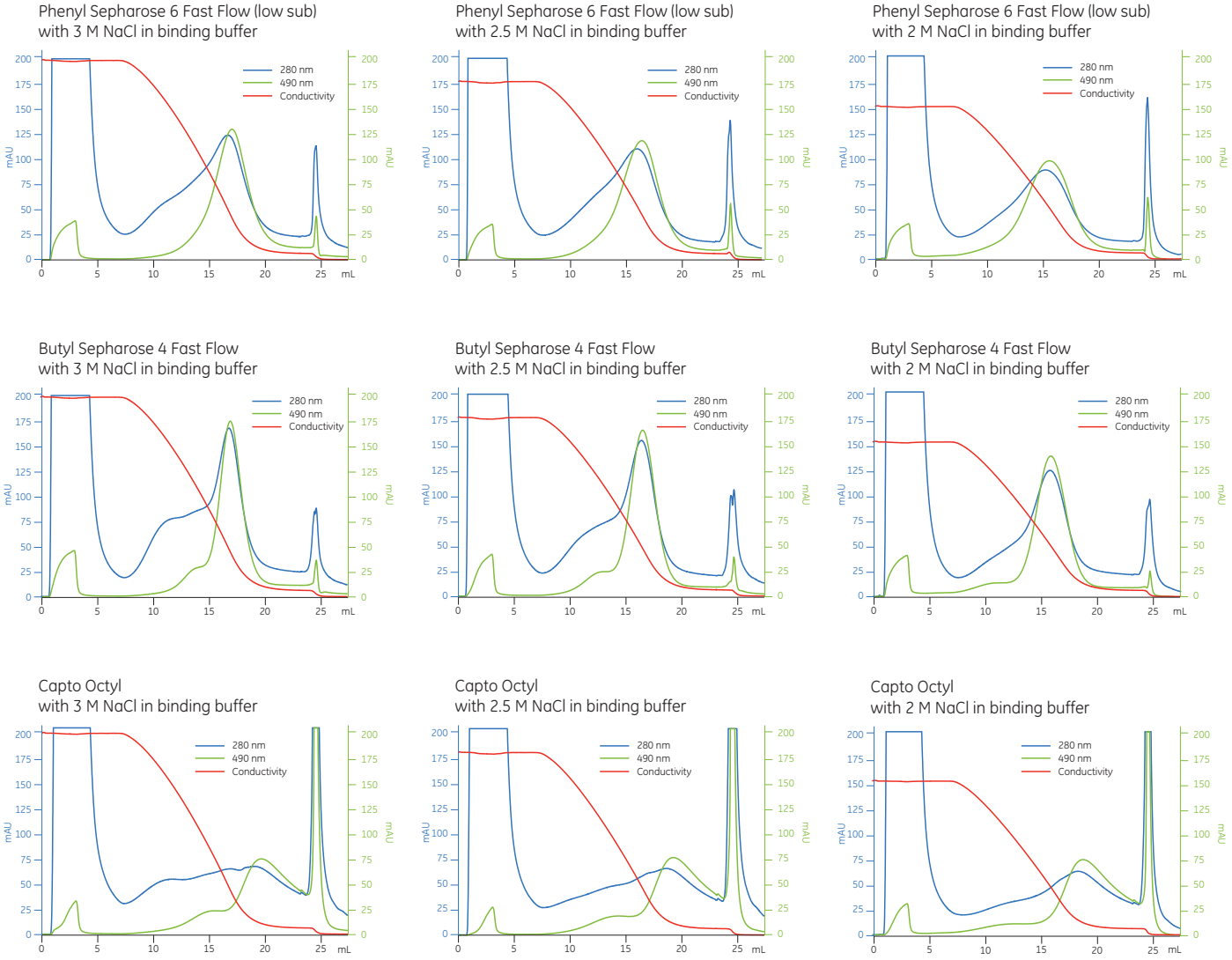

Fig 9. Chromatograms from Tricorn 5/50 column runs. The peak in the flowthrough is rGFP-related impurities and the sharp peak at the end of the chromatogram is strongly bound components that elute in 30% isopropanol.

Table 2. Dynamic binding capacities for three of the HIC media investigated

Medium	10% DBC, 2 M NaCl	10% DBC, 2.5 M NaCl	Difference
Octyl Sepharose 4 Fast Flow	3.4	4.3	21%
Butyl Sepharose 4 Fast Flow	4.9	5.1	4%
Phenyl Sepharose 6 Fast Flow (low sub)	6.0	6.2	3%

The binding capacity of a HIC medium is highly dependent on the properties of the target protein and impurities, the selectivity of the medium, and the binding conditions. It is expected to increase with the medium's hydrophobicity and the salt concentration, but that may also start to affect the yield. As expected, Butyl Sepharose 4 Fast Flow and Phenyl Sepharose 6 Fast Flow (low sub) show no significant difference in DBC between the two tested binding conditions.

The best candidates for purifying rGFP thus appear to be Butyl Sepharose 4 Fast Flow and Phenyl Sepharose 6 Fast Flow (low sub). They should therefore be further compared and optimized using the Design of Experiments tools in the ÄKTA platform.

Time and sample amounts consumed

Considerably less time and sample were used for screening experiments with the HIC PreDictor plates than with the conventional packed columns run in gradient mode†. Figure 10 shows the results; 77 mg sample protein vs 192 mg and 6 h vs 24 h respectively. The time for sample preparation and data evaluation is not included, but is about the same for both formats.

† **How the comparisons were made:** Four 96-well HIC PreDictor screening plates were used during this study and 200 µL sample was applied per well, i.e. a total of 76.8 mL sample (4 × 96 × 0.2 mL). If the same screening had been done on eight 1 ml HIC columns with 4 mL sample load per run, the first screening (with 3 different salt types) followed by the second screening (with 3 salt concentrations) would comprise 48 gradient runs in total. This works out at 192 ml sample for all column experiments. This estimation does not take into account that all experiments need an excess of sample. However, screening with 96-well filter plates requires approximately 2.5-fold less sample than traditional columns.

Moreover, the experimental time for the 96-well filter plates was approximately 6 h, compared with 24 h in total for 48 column experiments (a column run is approx. 30 min). We thus see a 4-fold difference between these two formats regarding experimental lead-time (the time from the experiment start to that when data are available to evaluate further).

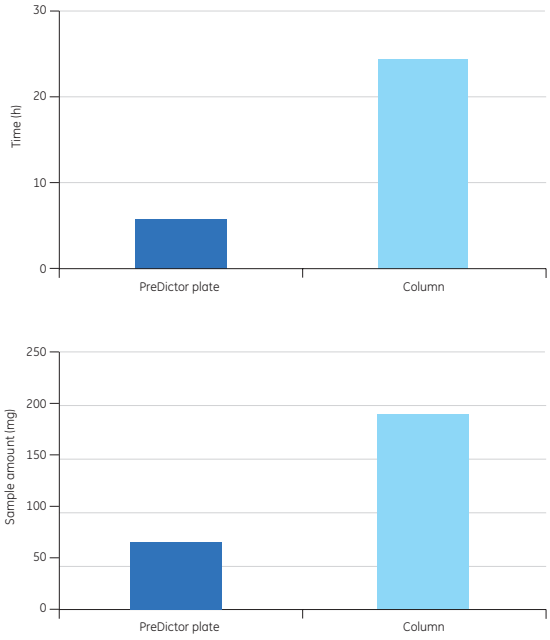

Fig 10. Estimate of time and sample consumption for PreDictor HIC screening plates compared with column chromatography (run in gradient mode) in Tricorn 5/50 columns packed with 1 mL of HIC medium.

Conclusion

Screening different HIC media, salt types and salt concentrations to find the best conditions to perform a capture step for rGFP expressed in *E. coli* using a clarified cell culture supernatant sample confirmed that plate experiments mirror chromatographic behavior in traditional columns, and that both time and sample can be saved in the early stages of process development.

The plates enabled rapid parallel screening of purification parameters for developing the capture step. Results indicated that both binding and elution conditions could be fine-tuned to obtain high purity of the target protein in the plate elutes. This approach proved that it is possible to rank different HIC media and predict the elution profile in a fast and effective way.

References

1. Hydrophobic Interaction and Reversed Phase Chromatography, Principles and Methods, 11-0012-69 AA
2. High-throughput Process Development with PreDictor plates, Principles and Methods, 28-9403-58 AA

Ordering information

Product	Code no.
PreDictor HIC Screening High Hydrophobicity, 50 µl	28-9923-97
PreDictor HIC Screening Low Hydrophobicity, 50 µl	28-9923-98
Assist 1.2 Software package	28-9969-17
Assist, 1-User e-license v1.2	28-9453-97

For more plates, columns and HIC media, see www.gelifesciences.com/predictor and www.gelifesciences.com/bioprocess

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