Rapid screening of a scalable, intermediate purification step for recombinant EGFP with HiTrap HIC Selection Kit

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CY13261-08May20-AN

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Abstract

We describe using the HiTrap™ HIC Selection Kit expanded with Butyl Sepharose™ High Performance (as HiTrap Butyl HP) to screen seven prepacked 1 ml HIC columns with different hydrophobicities. The aim of this screening was to determine the best combination of medium and running conditions for an intermediate purification step for recombinant green fluorescent protein (EGFP) according to set process goals. These goals included high resolution, and yield (at least 80%) and a dynamic binding capacity of at least 20 mg protein/ml medium, as well as scalability.

The screening strategy adopted proved successful and the resulting purification protocol was well-suited for scaling up to a robust large-scale process. Furthermore, adding HiTrap Butyl HP introduced a novel selectivity that will extend the usefulness of the kit for a broad range of applications.

These results, and the convenience with which they were obtained, demonstrate the value of the HiTrap HIC Selection Kit as a rapid and reliable means of screening HIC media and running conditions. The kit should prove equally useful for a wide range of other proteins suitable for this widely-used purification technique.

Introduction

Hydrophobic interaction chromatography (HIC), which separates molecules based on differences in their surface hydrophobicity, is well-established within protein purification today. By combining great versatility with high selectivity, it is mainly employed in the intermediate or final purification stages of a wide variety of substances.

Experimental goal

The aim of the study was to quickly develop an intermediate purification step for recombinant histidine-tagged, spectrum-shifted green fluorescent protein (EGFP) that met preset process performance goals of high resolution, high yield (at least 80%) and a dynamic binding capacity of at least 20 protein/ml medium. The purification protocol should also be easy to scale up.

Screening with HiTrap HIC Selection Kit

As several factors influence the interaction of proteins with HIC media, initial media and conditions screening is generally the best starting point for developing a purification protocol. By combining seven HIC media with different hydrophobic characteristics in a convenient prepacked column format, the expanded HiTrap HIC Selection Kit allowed us to quickly identify the best medium and suitable running conditions for it. When optimal conditions had been verified, the purification was scaled-up to a larger laboratory column. This serves as a starting point for developing a full-scale purification process.

Table 1 HiTrap HIC Selection Kit comprises seven prepacked HiTrap 1 ml columns containing different HIC media

Prepacked column	Medium	Ligand density (µmol/ml medium)	Main medium characteristics
HiTrap Butyl-S FF	Butyl-S Sepharose 6 Fast Flow[2]	10	Least hydrophobic of the HIC media. For binding and eluting relatively strong hydrophobic molecules at comparatively low salt concentrations. High throughput.
HiTrap Phenyl FF (low sub)	Phenyl Sepharose 6 Fast Flow (low sub)[2]	25	Broad hydrophobicity. Two ligand densities help find the best selectivity and capacity. High throughput.
HiTrap Phenyl FF (high sub)	Phenyl Sepharose 6 Fast Flow (high sub)[2]	40	
HiTrap Butyl FF	Butyl Sepharose 4 Fast Flow[2]	40	Low to medium hydrophobicity. Often works with relatively low salt concentrations. High throughput.
HiTrap Octyl FF	Octyl Sepharose 4 Fast Flow[2]	5	Hydrophobic character similar to phenyl and butyl ligands makes it an important complement to these other matrices. High throughput.
HiTrap Phenyl HP	Phenyl Sepharose High Performance[1]	25	Low to medium hydrophobicity. Selectivity similar to Phenyl Sepharose 6 Fast Flow. High resolution.
HiTrap Butyl HP	Butyl Sepharose High Performance[1]	50	Low hydrophobicity. Relatively high ligand density gives high dynamic binding capacity, even at relatively low salt concentrations. High resolution.

[1] Medium based on 34 µm matrix. Ideal for laboratory and process-scale purification where high resolution is needed.

[2] Medium based on 90 µm matrix. Ideal for laboratory and process-scale purification where high throughput is needed.

Materials and methods

Unless otherwise stated, all equipment and chromatographic media were from GE Healthcare (Uppsala, Sweden) and all chemicals used were of analytical grade.

Starting material

Green fluorescent protein (GFP) is a native protein found in the pacific jellyfish *Aequeuoria victoria*. Recombinant EGFP (Enhanced-GFP) expressed in *E. coli* has a molecular weight of 28 kDa and an isoelectric point of 6.2. A specific absorbance peak at 490 nm allows easy detection and quantification (see Results).

The EGFP used here was obtained as an eluate from an upstream ion exchange capture step on Capto™ Q (1). The sample was in a buffer containing 300 mM NaCl and 50 mM Tris-HCl, pH 8.2. EGFP content was 2.2 mg/ml as determined by spectrophotometric assay.

Initial media screening

HiTrap HIC Selection Kit comprises seven HiTrap 1 ml columns pre-packed with HIC media displaying different hydrophobic characteristics. Table 1 lists these media and their key attributes. All are suitable for production scale chromatography and thus available in larger HiPrep™ or HiLoad™ prepacked columns as well as in laboratory packs and bulk quantities, the latter as BioProcess™ chromatography media.

HIC is usually performed with moderate to high concentrations of salts in the start buffer, which promotes binding and helps stabilize the target protein. The bound molecule is then eluted by decreasing the salt concentration.

As the type and concentration of salt influences the behavior of proteins, each column in the kit was screened with different salts [$(NH_4)_2SO_4$, Na_2SO_4 and NaCl], each at two different concentrations [0.7 M and 1.7 M for $(NH_4)_2SO_4$ and Na_2SO_4; 2.0 M and 3.0 M for NaCl]. These combinations are based on what is common practice today for this type of chromatographic purification.

The seven columns (see Table 2) were connected to different column position valves in ÄKTAexplorer™ 100 system. Sample loading was 4.4 mg/ml and automated screening experiments were run using 50 mM Tris-HCl, pH 8.0, containing the six salt and concentration combinations stated above (i.e., 42 runs were made). Elution was performed with a 20 column volume (CV) gradient of 0% to 100% of 50 mM Tris-HCl, pH 8.0, at a flow rate of 1 ml/min.

Final selection, verification and initial scale-up

When the seven HIC media had been narrowed down to two candidates that best met the preset goals, their dynamic binding capacities (expressed as $QB_{10\%}$, see Table 3) were tested, each with two salt combinations. The final chosen combination was then verified and scaled-up in a Tricorn™

10/100 GL column (10 mm inner diameter) packed with 6.7 ml of the medium. To further optimize the method, the bed height was increased to 8.5 cm. Except for this change in bed height, all chromatographic conditions were the same as in the small-scale HiTrap 1 ml screening runs. The scaled-up protocol was run at two different linear fluid velocities (150 cm/h and 300 cm/h).

During the study, purities and recoveries were assessed by subjecting fractions of eluted peaks to analytical gel filtration and SDS-PAGE.

Results

Initial media screening

Monitoring the 490 nm-absorbing material showed that all seven HIC media separated the recombinant EGFP under the six different salt type and concentration combinations. Figure 1 illustrates chromatograms from screening all seven columns with 3 M NaCl in the start buffer. Different degrees of resolution of EGFP can be seen.

Several of the media resolved the 490 nm-absorbing material into two distinct peaks, indicating two forms of EGFP. Fractionation and further analysis by IMAC and ESI-MS analysis* revealed the smaller, less hydrophobic peak to have a molecular weight of 26.8 kDa and no binding to a HisTrap™ FF column (pre-packed with Ni Sepharose Fast Flow), while the larger peak had a molecular weight of 28.2 kDa and bound on HisTrap FF (data not shown). This is consistent with the smaller peak being a truncated version of the EGFP-construct with a sequence of 12 amino acids (LAAALEHHHHHH) cleaved from the C-terminus.

The overall screening results of seven different HiTrap HIC columns (42 experimental runs) were assessed in terms of EGFP binding, peak shape, resolution and purity. The outcome varied considerably but was generally in line with that expected from the media characteristics listed in Table 1. For example, high salt concentration seems to bind the protein too strongly to Phenyl Sepharose Fast Flow (high sub) so that the protein did not elute fully within the gradient (Fig 1 C). The two media based on a 34 µm-sized matrix (Butyl Sepharose High Performance and Phenyl Sepharose High Performance) with starting buffer containing salt concentrations of 3.0 M NaCl and 0.7 M $(NH_4)_2SO_4$ respectively eluted EGFP protein (as indicated by the green curve) with good resolution in well-defined peaks. Figure 1 F and G illustrate these separations with 3.0 M NaCl (See Table 2) in the start buffer. Both media were therefore selected and subjected to further screening regarding binding capacity.

* ESI-MS analysis was done on an Agilent™ 1100 LC/MSD Trap SL ion trap mass spectrometer (Agilent Technologies). Samples were diluted to 0.04 to 2 µM in 1% formic acid and injected into the electrospray instrument run in positive ion mode.

Columns: HiTrap HIC Selection Kit (7 x 1 ml): HiTrap Phenyl HP, HiTrap Butyl HP, HiTrap Phenyl FF (low sub), HiTrap Phenyl FF (high sub), HiTrap Butyl FF, HiTrap Octyl FF)
Sample: 2 ml (4.4 mg/ml) EGFP
Start buffer (A): 50 mM Tris-HCl, pH 8.0, containing 3.0 M NaCl
Elution buffer (B): 50 mM Tris-HCl, pH 8.0
Flow rate: 1 ml/min (150 cm/h)
Gradient: 20 CV linear gradient from 0% to 100% elution buffer

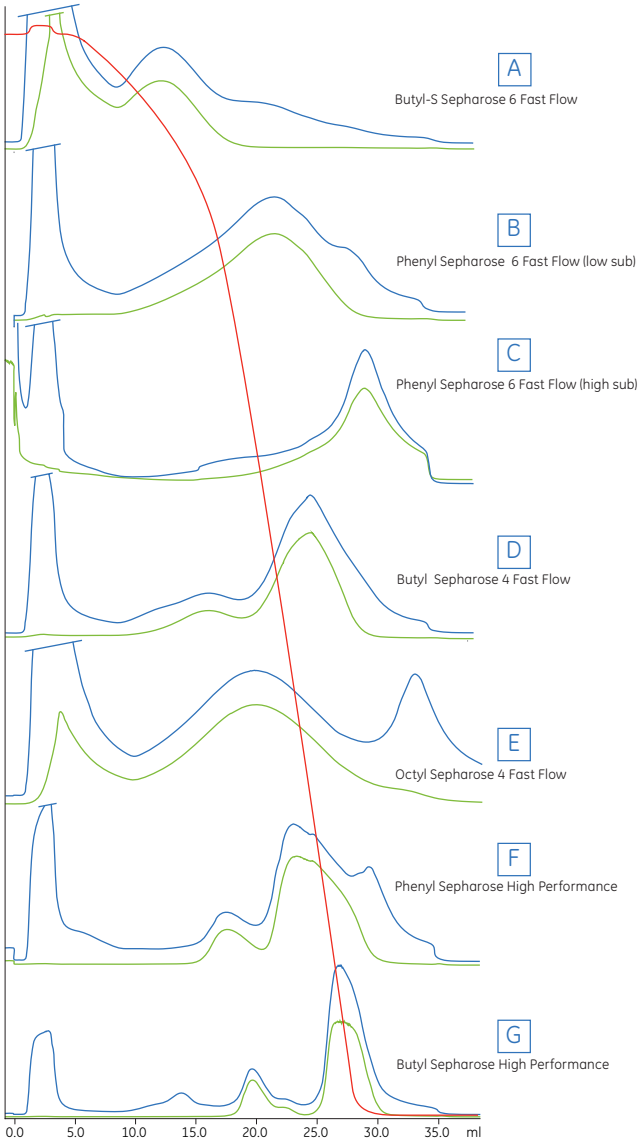


Fig 1. Chromatograms from screening runs on the seven different media in HiTrap HIC Selection Kit columns. Blue = 280 nm absorbance, green = 490 nm absorbance. The start buffer contained 3.0 M NaCl in all these runs.

Table 2. The layout for the broad initial screening comprises testing all seven media in the HIC HiTrap Selection Kit using three different salts at two concentrations each, 42 runs in total (Qualitative estimation)

Medium/salt	Butyl S FF			Phenyl FF low sub			Octyl FF			Phenyl FF high sub			Phenyl HP			Butyl FF			Butyl HP		
	Bi	Rs	Pu	Bi	Rs	Pu	Bi	Rs	Pu	Bi	Rs	Pu	Bi	Rs	Pu	Bi	Rs	Pu	Bi	Rs	Pu
1.7 M $(NH4)_2SO_4$	H	M	M	H	M	M	H	H	M	H	H	H	H	H	H	H	H	H	H	H	H
0.7 M $(NH4)_2SO_4$	L	L	L	M	L	H	M	L	L	H	L	M	H	H	H	M	M	H	H	H	H
1.7 M Na_2SO_4	H	M	H	H	M	L	H	H	M	H	L	M	H	H	H	H	H	H	H	H	H
0.7 M Na_2SO_4	L	L	L	H	M	L	H	H	M	H	L	L	H	H	H	H	H	H	H	H	H
3.0 M NaCl	M	L	L	H	M	L	M	H	M	H	L	M	H	H	H	H	H	H	H	H	H
2.0 M NaCl	L	L	L	M	L	M	L	L	L	H	L	H	H	M	H	M	M	M	H	H	H

Bi = Binding Rs = Resolution Pu = Purity

H = High M = Medium L = Low

Dynamic binding capacity

Dynamic binding capacities for EGFP at at a flow rate of 1 ml/min (150 cm/h, residence time 1 min) were determined for the two selected media in NaCl and $(NH_4)_2SO_4$ at concentrations of 3.0 M and 0.7 M respectively. Table 3 shows the results.

Screening data clearly show that higher capacities were obtained with sodium chloride than with ammonium sulfate at the chosen salt concentrations. Furthermore, Butyl Sepharose High Performance had the highest dynamic capacity (32 mg/ml medium) at 3.0 M NaCl.

Table 3. Dynamic binding capacities for EGFP on Butyl Sepharose High Performance $QB_{10\%}$ for EGFP and Phenyl Sepharose High Performance

	$QB_{10\%}$ (mg/ml)[1]	
Medium	3.0 M NaCl	0.7 M $(NH_4)_2SO_4$
Butyl Sepharose High Performance	32.0	23.4
Phenyl Sepharose High Performance	23.9	16.8

[1] $QB_{10\%}$ is a measurement of dynamic bed capacity defined as the amount of protein loaded onto a chromatography medium at 10% breakthrough based on protein load absorbance, in this case measured as absorbance at 490 nm.

Verification

As a result the binding capacity test, the preliminary purification protocol chosen was Butyl Sepharose High Performance with 50 mM Tris-HCl, pH 8.0, containing 3.0 M NaCl as start buffer. However, the highest dynamic capacity obtained (32 mg/ml medium) was much greater than the goal set for the process (20 mg/ml). A capacity of 32 mg/ml is too high to deliver a robust purification since the target product is likely to leak during the washing step.

To achieve a greater margin of safety, column loading was limited to 70% of the $QB_{10\%}$. A gradient purification procedure was thus designed based on applying 22 mg EGFP to the HiTrap Butyl HP column.

To secure maximum EGFP binding at this load, the sample was applied with the column equilibrated with 50 mM Tris-HCl, pH 8.0, containing 3.3 M NaCl. This slight increase in salt concentration gives a conductivity equal to the sample. Figure 2 shows the overall running conditions and the chromatogram obtained. The peak is clearly defined in the elution gradient. Furthermore, EGFP leakage when unbound material is washed from the column prior to elution is very low.

Scale-up

A Tricorn 10/100 GL column (10 mm inner diameter) packed with 6.7 ml of Butyl Sepharose High Performance was used for the scale-up experiment. Bed height was increased from 2.5 cm to 8.5 cm and the protocol was run at two linear flow velocities (150 cm/h and 300 cm/h), the latter being the maximum recommended for Sepharose High Performance media.

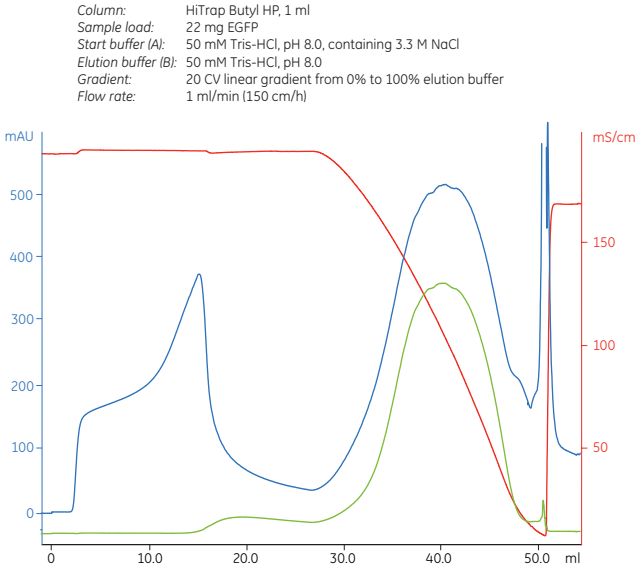


Fig 2. Verified purification on HiTrap Butyl HP with 22 mg EGFP sample loading (70% of the dynamic binding capacity) and start buffer containing 3.3 M NaCl. Blue = $QB_{10\%}$ 280 nm absorbance, green = 490 nm absorbance.

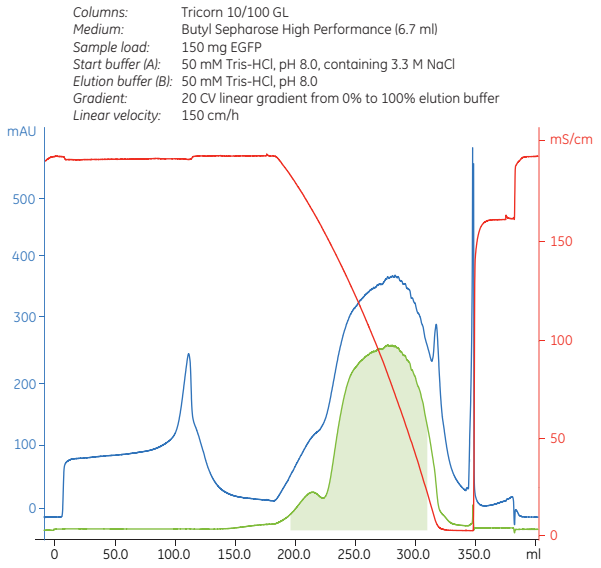
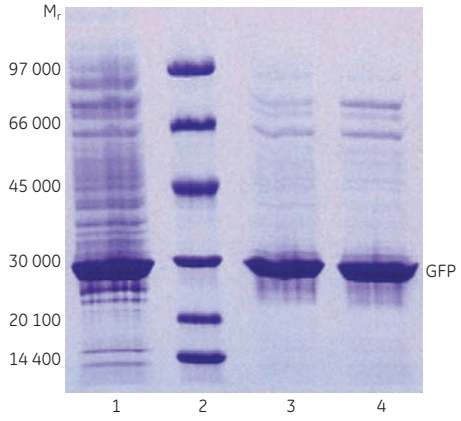


Fig 3. The scaled-up purification on Tricorn 10/100 GL. The peak denoted by the green shaded peak had a purity of 92% as measured by analytical gel filtration. Blue = 280 nm absorbance, green = 490 nm absorbance.



Lanes

1. Starting material diluted 1:4
2. LMW (low molecular weight marker)
3. Pool from 1 ml HiTrap Butyl HP diluted 1:4
4. Pool from Tricorn 10/100 GL packed with Butyl Sepharose High performance diluted 1:4

Fig 5. SDS-PAGE analysis of fractions from the screening/scale-up runs.

Figure 3 shows a result of the 150 cm/h run, where a small "pre-peak" is evident. This is consistent with the previously identified truncated EGFP variant (see page 3) and probably reflects better resolution due to the greater bed height in Tricorn 10/100 GL compared to HiTrap 1 ml.

EGFP purity and recovery in the pooled fractions were analyzed by gel filtration and SDS-PAGE. Recovery was calculated using the area of the 490 nm curve between 15.5 and 21 ml retention volume. The results of both analyses correlated well and are shown in Figures 4 and 5 respectively. Table 4 summarizes purity and recovery data from different stages of the study.

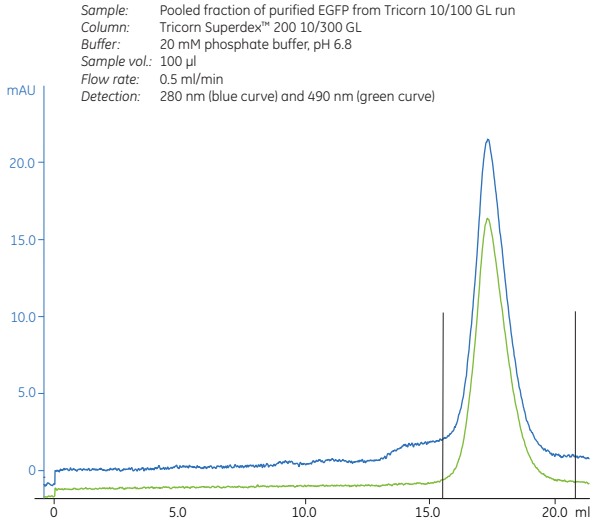


Fig 4. Analytical gel filtration of the pooled EGFP fraction from the Tricorn 10/100 GL column demonstrates high purity. See also Figure 5 and Table 3.

Table 4. EGFP purity and recovery assessed by analytical gel filtration

Fraction	Purity (%)	Recovery (%)
Starting material (captured on Capto Q)	77	—
Pool from HiTrap Butyl HP	91	90
Pool from Tricorn 10/100 GL	92	86

Conclusions

The strategy adopted to develop an intermediate-stage HIC purification for recombinant EGFP using the expanded HiTrap HIC Selection Kit gave the desired result. The set process goals regarding resolution, yield and capacity were all met. Furthermore, automatic screening on ÄKTAexplorer 100 system meant that this result was achieved with a high degree of speed and convenience.

Butyl Sepharose High Performance and Phenyl Sepharose High Performance were quickly identified as the best candidates, allowing further dynamic binding capacity tests to be run on just these two media. The former was then judged to be the best alternative. Purification verification on Butyl Sepharose High Performance and subsequent scale-up on a Tricorn column confirmed that the developed purification was both robust and scaleable.

HiTrap HIC Selection Kit thus yielded a scalable intermediate purification step in a limited number of test runs. The overall screening method was effective and easy to perform, and should be broadly applicable to many other proteins. In this particular purification, HiTrap Butyl HP gave the best result.

Reference

1. Application note: High productivity capture of Green Fluorescent Protein on Capto Q, GE Healthcare, 11-0026-20, Edition AB, (2005).

Ordering information

Product	Quantity	Code no.
HiTrap HIC Selection Kit	7 x 1 ml	28-4110-07
HiTrap Phenyl FF (high sub)	5 x 1 ml	17-1355-01
HiTrap Phenyl FF (high sub)	5 x 5 ml	17-5193-01
HiTrap Phenyl FF (low sub)	5 x 1 ml	17-1353-01
HiTrap Phenyl FF (low sub)	5 x 5 ml	17-5194-01
HiTrap Phenyl HP	5 x 1 ml	17-1351-01
HiTrap Phenyl HP	5 x 5 ml	17-5195-01
HiTrap Octyl FF	5 x 1 ml	17-1359-01
HiTrap Octyl FF	5 x 5 ml	17-5196-01
HiTrap Butyl FF	5 x 1 ml	17-1357-01
HiTrap Butyl FF	5 x 5 ml	17-5197-01
HiTrap Butyl-S FF	5 x 1 ml	17-0978-13
HiTrap Butyl-S FF	5 x 5 ml	17-0978-14
HiTrap Butyl HP	5 x 1 ml	28-4110-01
HiTrap Butyl HP	5 x 5 ml	28-4110-05

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28-9192-12 AB 03/2009