Application Note

Scouting Protein Purification Conditions Using Vivapure Centrifugal Ion Exchange Membrane Absorbers

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Introduction
For separation and purification of proteins from biological samples, different characteristics of the target protein e.g. its size, charge, hydrophobicity or specifically engineered tags are exploit.

With ion exchange chromatography, separation is achieved on the basis of different charges of biomolecules. This makes it to a versatile method often used for pre-fractionation or purification of a target protein from crude protein mixtures. To optimize the purification procedure for an individual target, several binding and elution conditions have to be tested on cation and anion exchange matrices.

In contrast to traditional column chromatography methods, Vivapure IEX centrifugal columns allow scouting of several chromatography conditions in parallel, leading quickly to different fractions which can be further analyzed for enriched or even already purified target protein.

Here, we demonstrate the performance of Vivapure IEX Mini spin columns for evaluation of optimal purification conditions of cloned SH2 domains from an *E. coli* lysate in a two step procedure. This protocol can generally be employed for finding a purification method based on ion exchange chromatography for a given target protein as it is fast and only uses up small amounts of the sample.

In the first step of this protocol, binding conditions are evaluated by loading the sample on Vivapure Q and S columns at various pH-values, eluting bound proteins with a high salt concentration buffer and analyzing all fractions for the target protein. This step results in the optimal binding pH and the best ion exchange chemistry for the purification.

In a second step, the best elution method is evaluated by applying increasing salt concentrations to columns which were shown to bind the target protein in step one, leading to a complete purification protocol in less than one hour.

Experiment
Using the described scouting procedure, a purification method for a SH2 domain expressed in *E. coli* was developed. In a first step, proteins were bound to the Vivapure IEX membranes at different pH values, then eluted with high-salt buffer. In Step Two a fresh sample was adjusted to the respective pH elucidated previously as the best choice for binding the protein and was loaded onto a new column for refining optimal elution conditions.

Materials
- Vivapure Mini Q H spin columns
- Vivapure Mini S H spin columns
- Minisart syringe filter (0.45 µm CA, Sartorius AG)
- Centrifuge, 45°-fixed-angle rotor; 2000 × g

Buffers used

Buffer A:	25 mM Citrate, pH 4
Buffer B:	25 mM Potassiumphosphate, pH 6
Buffer C:	25 mM HEPES, pH 8
Buffer D:	25 mM Sodiumbicarbonate, pH 10
Buffer E:	25 mM Citrate, pH 4, supplemented with 1 M NaCl.
Buffer F:	25 mM Potassiumphosphate, pH 6, supplemented with 0.2 M, 0.4 mM, 0.6 mM, 0.8 mM, & 1 M NaCl, respectively.
Buffer G:	25 mM HEPES, pH 8, supplemented with 1 M NaCl
Buffer H:	25 mM Sodiumbicarbonate, pH 10, supplemented with 1 M NaCl

Procedure

Step One: Scouting for binding conditions to the appropriate ion exchange chemistry.

Expression of target protein
300 ml LB media were inoculated with 4 ml of an overnight culture and incubated at 37°C, shaking at 150 rpm until an OD600 of 1.0 was reached. IPTG was added to a final concentration of 1 mM and incubated for further 4 h with shaking at 150 rpm. Cells were harvested by centrifugation at 4000 × g for 30 min at 4°C. The pellet was resuspended in 35 ml PBS (150 mM KPi, pH 7,3) and cells were lysed by addition of lysozyme to a final concentration of 0.1 mg/ml and incubation for 1 h at 37°C. Insoluble particles as cell debris were removed by centrifugation at 10000 × g for 30 min at 4°C.

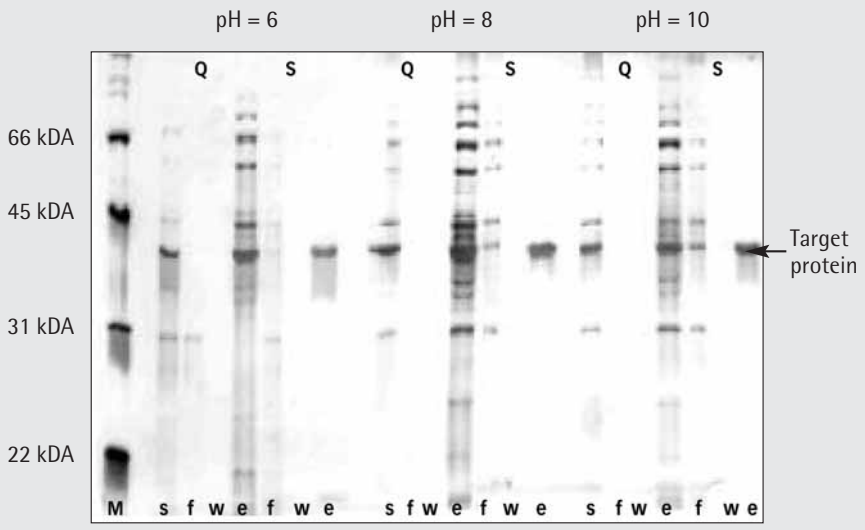
Sample preparation
4 × 200 µl of the cell lysate were diluted with 1.8 ml binding buffer A to D each, to adjust the sample to the respective pH conditions. In order to avoid clogging of the membranes in the Vivapure Mini spin columns, samples were clarified by passage through Minisart syringe filters.

Column equilibration
4 × Q and 4 × S Vivapure Mini spin columns were labeled 4, 6, 8 and 10 corresponding to the pH of the buffer to be used. To each spin column, 400 µl of the corresponding binding buffer were added and spun for 5 minutes at 2000 × g.

Binding and washing
400 µl of the clarified samples adjusted to pH values 4, 6, 8 and 10 were applied each to the correspondingly equilibrated Vivapure Q and S spin columns. Columns were spun for 5 min at 2000 × g.

Afterwards, Vivapure Mini spin columns were reloaded with 400 µl sample and spun again for 5 min at 2000 × g. Loosely bound proteins were washed away with the application of 400 µl of the respective binding buffer to each of the columns and spinning for 5 min at 2000 × g. Flow-through and wash fractions were collected for subsequent detection of the target protein.

Complete elution of bound proteins
200 µl of elution buffer E, F, G and H, were applied to the washed columns and spun for 3 min at 2000 × g. Eluates were saved for subsequent analysis.



Sample	Sample volume (l)	Volume loaded on the gel (l)
M = Broad range marker		
s = Sample before application	800	4
f = Flow-through	800	4
w = Wash fraction	400	4
e = Elution with 1 M NaCl	200	4

Fig. 1: Scouting for optimal binding conditions of a SH2 domain expressed in *E. coli*. SDS gel (reducing, 12%), silver stained. Shown are sample before loading, flow-through, wash, and elution fractions (1 M NaCl) from Vivapure Q and S Mini spin columns, at the various pH values tested.

Analysis
4 µl of flow-through, wash, and elution fractions from each column were analyzed on reducing SDS-PAGE followed by silver staining.

Result of Step One
Dilution of the *E. coli* lysate with binding buffer A (25 mM Citrate, pH 4) lead to complete precipitation of sample proteins. Thus, pH 4 could not be tested in this experiment. As can be seen on the SDS gel in figure 1, the target protein was present in the eluate of the Vivapure Q Mini spin column at all pH values tested together with most of the *E. coli* proteins (Lanes Q "e"). In contrast, using the Vivapure S Mini spin column, at all pH-values tested, most *E. coli* proteins did not bind to the membrane and were found in the flow-through (Lane Lane S "f"), thus resulting in pure target protein in all elution fractions (Lane S "e").

Differences could be detected in the binding efficiency of the target protein as at pH 8 traces of the target protein were already found in the flow-through, with slightly higher amounts at pH 10 (Lane S "e"). At pH 6, the most efficient binding of the target protein to the S membrane was observed. Now that the binding conditions, i. e. binding pH and the best suited ion exchange chemistry, were found, the elution protocol of the target protein was optimized in a second step.

Step Two: Optimizing elution conditions

Sample preparation
Taking account of the results of Step One, 200 µl cell lysate were diluted with 1.8 ml binding buffer B (25 mM KPi, pH 6). In order to avoid clogging of the membrane in the Vivapure Mini spin column, the pH adjusted sample was clarified by passage through a Minisart syringe filter.

Column equilibration
400 µl binding buffer B were applied to one Vivapure S Mini spin column and spun for 5 minutes at 2000 × g.

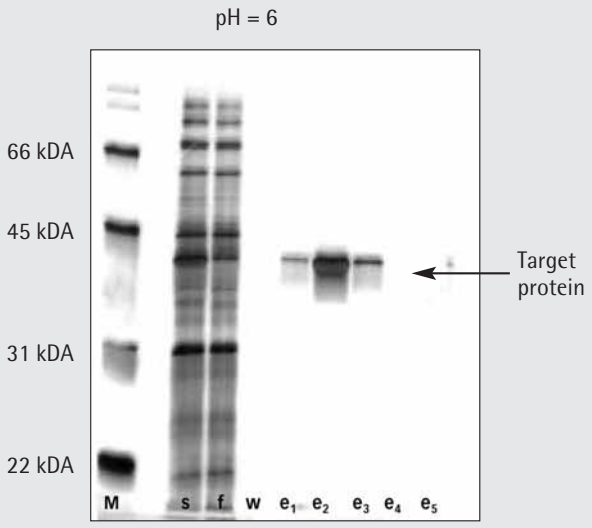
Binding and washing
400 µl of the clarified sample were applied to the equilibrated Vivapure S column and spun for 5 min at 2000 × g. Afterwards, the Vivapure S Mini spin column was reloaded with 400 µl sample and spun again for 5 min at 2000 × g.

Loosely bound proteins were washed away by application of 400 µl binding buffer to the column and spinning for 5 min at 2000 × g. Flow-through and wash fraction were saved for analysis.

Stepwise elution
100 µl elution buffer F, supplemented with 0.2 M NaCl were applied to the Vivapure S Mini spin column and spun for 3 min at 2000 × g. The eluate was collected. In the next step, 100 µl of elution buffer F, supplemented with 0.4 M salt were applied and again spun for 3 min at 2000 × g. Elution was continued until the entire gradient had been tested, saving the eluates from each step.

Analysis
4 µl of flow-through, wash, and elution fractions from each column were analyzed on reducing SDS-PAGE followed by silver staining.

Result of Step Two
The target protein started to elute with 200 mM NaCl, however the main fraction eluted with 400 mM NaCl. Traces of the target protein were also found in the next elution step with 600 mM NaCl, but this might be due to the low elution volume.



Sample	Sample volume (l)	Volume loaded on the gel (l)
M = Broad range marker		
s = Sample before application	800	16
f = Flow-through	800	16
w = Wash fraction	400	16
e1 = 25 mM KPi, pH 6, 200 mM NaCl	100	8
e2 = 25 mM KPi, pH 6, 400 mM NaCl	100	8
e3 = 25 mM KPi, pH 6, 600 mM NaCl	100	8
e4 = 25 mM KPi, pH 6, 800 mM NaCl	100	8
e5 = 25 mM KPi, pH 6, 1 M NaCl	100	8

Fig. 2 Scouting for optimal elution conditions of a SH2 domain expressed in *E. coli*. SDS gel (reducing, 12 %), silver stained. Sample before loading, flow-through, wash, and elution fractions from Vivapure S Mini spin column at pH 6 are shown.

Discussion
A two-step procedure was used to rapidly scout optimal purification conditions for a target protein (a SH2 domain from *E. coli* lysate) with ion exchange chromatography. In the first step, the most suited buffer pH for binding the target protein to the most adequate ion exchanger was verified. In the second step, the elution condition was optimized building on the results gained in step one of this protocol (elution optimization after optimal binding of the target to the proper ion exchanger). With the scouting procedure described here, it was possible to quickly and conveniently purify the target protein to homogeneity. The results obtained in this experiment can be used for various ends, e.g:

– polishing a specific protein after a first chromatography step with another chemistry

– establishing quickly a FPLC method for a new protein

– finding a purification method for a new protein for upscaling with Vivapure Maxi or Mega.

For these purposes Vivawell 96well plates, Vivapure Maxi and Mega columns and Sartobind membrane adsorber units with FPLC connectors are available.

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