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Vivaflow® and Vivaspin® Workflow in Protein Research Laboratories

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Application Note #04

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Concentration and Purification of Proteins in Cell Culture Supernatant Using Sartorius Vivaflow®, Vivaspin® and Vivapure® Products

This protocol demonstrates how the Vivaflow® cassettes, Vivapure® Ion Exchange spin columns and Vivaspin® devices can be used in order to perform a complete protein purification workflow, from concentration and diafiltration of the original protein source, a cell culture supernatant, to final concentration | desalting of the purified protein. This protocol shows in detail the recoveries after each step along with the time needed for every purification and concentration step.

Efficiency and efficacy of a multiple cycle experimental procedure was performed using Vivaflow® tangential flow cassettes for initial concentration and diafiltration of a cell culture supernatant, followed by Vivapure® Ion Exchange spin columns for the protein purification step and finally Vivaspin® 20 ultrafiltration devices for the final sample concentration and desalting. An artificial mixture of proteins in a RPMI-1640 culture medium was created to mimic the type of product that many researchers culture using e.g. the UniVessel device. This procedure further reflects a method that can be adapted to a large number of protein purification protocols, adapting MWCOs and device sizes where necessary.

Part 1 – Creating and concentrating the culture medium

2 bottles (4 g) of RPMI-1640 were dissolved into 1.8 l dd-H_2O and 4 g of Sodium Acetate was added.

The pH was adjusted to 7.2 using 1M HCl. 2 g of BSA and 1 g of Lysozyme was added as protein samples, meant to be separated by chromatography. The volume of the cell culture supernatant sample was brought up to 2 l using dd-H_2O. After every preparation, concentration and purification step, 1 ml sample was set aside for SDS gel analysis at the end of the preparation.

Ion Exchange chromatography was chosen as the method of choice for purifying lysozyme from the cell culture supernatant, especially from the "contaminant" BSA. For this, the 2 l cell culture supernatant needed to be concentrated and then diafiltered to adjust the sample to the starting conditions needed for the ion exchange chromatography binding step.

For concentration and diafiltration, the Vivaflow® 200 was used with a 5 kDa PES membrane. Vivaflow® 200 is a ready-to-use laboratory crossflow cassette in an acrylic housing, which allows caustic cleaning and 4-5 re-uses. Two cassettes can be run in parallel for the concentration of up to 5 l sample volumes. For the 2 l sample to be concentrated in this experiment, one cassette was sufficient. A Masterflex pump with an Easy Load, size 16 pump head was used to run the Vivaflow® 200 cassette. Figure 1a. and 1 b. show the Vivaflow® 200 set up before and during the concentration process.

The Vivaflow® 200 system was set up and run at 3 bar. Once 1.8 l of filtrate had been collected, the pump was stopped, the tubes removed from the cell culture medium concentrate and filtrate and the Vivaflow® system was purged with dd-H_2O. This solution now contained a 10 fold concentration of the constituent proteins from the original culture-medium.

A BCA protein detection test conveyed a 100% recovery of protein after this first concentration step. Table 1 indicates the time needed for the sample concentration.

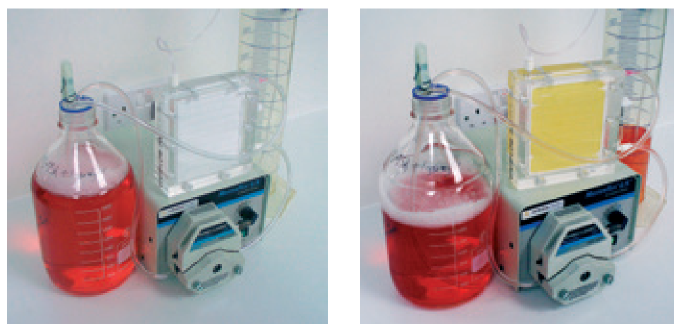

Fig. 1a. and 1 b: Vivaflow® 200 set up before (1a) and during (1b) the sample concentration process.

Vivaflow® 200 (5kDa MWCO)

Filtrate Volume (ml)	Time taken (hr:min:secs)
0	0:00:00
100	0:03:16
200	0:06:50
300	0:10:45
400	0:14:38
500	0:18:36
600	0:22:43
700	0:26:57
800	0:31:14
900	0:36:01
1000	0:40:50
1100	0:45:46
1200	0:50:36
1300	0:55:32
1400	1:00:24
1500	1:05:26
1600	1:10:28
1700	1:15:52
1800	1:21:50

Table 1: Vivaflow® 200, PES, 5 kDa MWCO concentration speed.

Part 2 – Buffer exchange of culture medium concentrate
The Vivaflow® 200 system was used for fast and easy diafiltration. To this end, the diafiltration cup, a Vivaflow® accessory, was filled with the 200 ml concentrated sample. Figure 2 shows the diafiltration set up. The Vivaflow® 200 system was set up as before, however attaching an additional tube to the diafiltration lid and placing this new inlet tube into a 25 mM Sodium Acetate (pH 5.5) buffer (needed to re-adjust the sample concentrate for the ionic starting conditions of the ion exchange chromatography step which was to follow). This leads to the concentration of the sample in the reservoir and to the extent in which the original buffer is removed and collected as waste (filtrate), new buffer (25 mM Sodium Acetate) is sucked into the closed system, gradually leading to a buffer exchange while keeping the sample volume constant at 200 ml. The system was run at 3 bar. Once 1 l of buffer had been exchanged, the filtration was stopped.
The 200 ml solution now contained the correct buffer to maintain the stability of the proteins of interest for the next part of the protocol and had the correct pH and salt concentration for the ion exchange binding step. BCA protein quantification again showed a 100% protein recovery.

Table 2 shows the time needed for diafiltration of 200 ml sample against 1000 ml exchange buffer, again using Vivaflow® 200 with a 5 kDa PES membrane.

Fig. 2: Diafiltration system set up for buffer exchange. Culture medium concentrate can be seen in the center of the image. 1 L 25 mM Sodium Acetate (exchange buffer) can be seen connected to the system on the left of the image.

Filtrate Volume (ml)	Time taken (hr:min:secs)
0	0:00:00
100	0:06:58
200	0:14:16
300	0:22:39
400	0:29:40
500	0:37:02
600	0:44:15
700	0:51:34
800	0:58:54
900	1:06:03
1000	1:13:02

Table 2: Diafiltration of 200 ml concentrated cell culture supernatant containing the proteins lysozyme and BSA against 1000 ml 25 mM Sodium Acetate.

Part 3 – Purification of Lysozyme, the protein of interest
The purification of lysozyme was performed using a Vivapure® cation exchange membrane adsorber devices (Vivapure® Maxi H S). The membrane adsorber matrix holds the active ligands and performs like a traditional cation exchanger. Membrane adsorbers represent a special form of chromatography matrix. Unlike traditional chromatography resins, they make use of convective transport to bring proteins to the ion exchange surface; hence, binding, washing and elution is performed quickly and high binding capacities are even achieved at high flow rates. This allows the use of the chromatography matrix in fast and convenient centrifugal spin columns (Fig. 3).

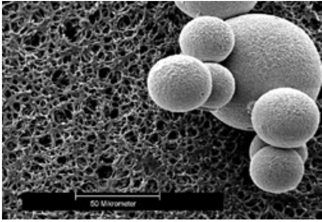

Fig. 3: The electron microscopic image of chromatography gel beads (upper right) in comparison to a Q ion exchange membrane adsorber (background) reveals 100 fold larger pore sizes of the membrane adsorber.

Two Vivapure® Maxi H S type devices (Fig. 4) were equilibrated with 10 ml of 25 mM Sodium Acetate, pH 5.5 each, by filling with 10 ml of this buffer and centrifuging for 5 min. in a swing bucket centrifuge at 500 xg and discarding the flow through.
Using the concentrated and buffer exchanged sample from Part 2, 10 ml sample were pipetted into each of these two equilibrated Vivapure® devices and centrifuged again for 5 min. in a swing bucket centrifuge at 500 xg. The Vivapure® devices were washed with further 10 ml of 25 mM Sodium Acetate, discarding the flow through, followed by an elution step with 5 ml of 1 M NaCl in 25 mM Sodium. A BCA test revealed a 95 % lysozyme recovery.

Fig. 4: Vivapure® Maxi spin columns can be used in a centrifuge for fast and easy protein purification.

The eluate was then concentrated in a Vivaspin® 20 (PES, 5 kDa MWCO), Figure 5., and centrifuged at 5000 xg for 10 min. or until approximately 2 ml of concentrate had been collected. The device was then re-filled with 18ml 50mM Potassium Phosphate buffer, pH 7.2 to 20 ml for a final buffer exchange and desalting of the purified sample. The sample was again centrifuged until a final sample volume of 2 ml had been attained. A BCA test revealed a 97 % lysozyme recovery.

Fig. 5: Vivaspin® 20 ultrafiltration device, on the right with a pressure cap which allows pressurization of the device as well and the regular utilization in a centrifuge.

Part 4 – Analyzing the samples
The samples of the individual steps were analyzed by SDS gel, using reducing sample buffer (prepared by adding 50 µl 2-mercaptoethanol to 950µl Laemmli sample buffer). For all steps, 5µl of the 1 ml sample taken during the experiment were diluted with 95µl reducing sample buffer, of which 20 µl were loaded onto a 12% tris-HCI SDS gel (Fig. 6)

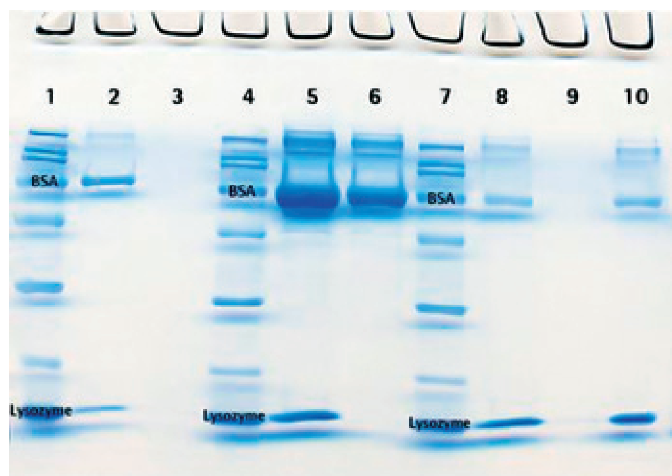

Fig. 6: Coomassie stained 12% tris-HCI SDS gel loaded with 20 µl sample preparations. Lane 1: Marker (SDS Broad range marker); Lane 2: Original sample; Lane 3: Original sample filtrate (Part 1); Lane 4: Marker; Lane 5: Buffer exchange concentrate (Part 2); Lane 6: Filtrate after binding (Part 3); Lane 7: Marker; Lane 8: Filtrate after eluting (Part 3); Lane 9: Filtrate after concentrating and desalting (Part 3); Lane 10: Concentrate after concentrating and desalting.

Conclusion
The overall result shows that a standard and straightforward procedure can be followed to concentrate, purify, isolate and analyze a protein of interest from a cell culturing device, using Vivaflow® 200 tangential flow units for cell culture supernatant concentration and diafiltration, Vivapure® for ion exchange chromatography followed by Vivaspin® 20 for final sample concentration and desalting.

In many cases dialysis, which is an overnight procedure would be performed instead of the much quicker alternative ultrafiltration. Here, we show how time saving and efficient ultrafiltration is for diafiltration and desalting applications, as well as for protein concentration.

The complete set up and completion of protein purification takes approx. 3.45 h using this method, starting form a culture supernatant, with high protein recoveries in each step (see Table 3)
The total protein purification procedure can be completed within 1 working day, including SDS gel analysis, utilizing this time saving strategy, when adapted to individual needs.

Task	Time	Recovery
Vivaflow® 200 set up and run through	1 hour 25 min.	100%
Vivaflow® 200 Diafiltration set up and run through	1 hour 20 min.	100%
Vivapure® purification	45 min.	95%
Vivaspin® Lysozyme desalting | concentration	30 min.	97%
Total	3 hours 45 min.	92%

Table 3

Products used in this experiment	**order No.**
Vivaflow® 200, PES, 5kDa	VF20P1
500 ml Diafiltration cup	VFA006
Vivapure® S H Maxi	VS-IX20SH08
Vivaspin® 20, 5 kDa	VS2011

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