HFE purification (method by José Lebrón)

This procedure has been optimized for purifying the mutant and wild-type forms of HFE from CHO cell supernatants using two types of columns—an immunoaffinity column and a size exclusion column. If the supernatant has been recently filtered (about a week), then it is not necessary to re-filter it; otherwise, re-filter it before running through the column. A liter of HFE-containing cell supernatant from cells grown on plates will typically yield around 1-5 mg. The yield from cell pharm cell supernatants is approximately 20-40 mg/liter. This information is important in trying to determine how much supernatant should be run through the column. I estimate that the capacity of the 1C3 anti-HFE column is about 2-3 mg. If purifying HFE from plate-derived supernatant I normally run one liter at a time. But if I am purifying HFE from cell pharm-derived supernatant then I run about 75 ml at a time. Currently, there is no wild-type HFE supernatant left (derived from either source). There is, however, a box in the cold room containing the supernatants from some of the HFE mutants.

First purification step—Immunoaffinity column

The buffers I use for this step are:

> Washing buffer—50 mM Tris-Cl pH 7.4, 150 mM NaCl, 0.05% Na-azide
> Elution buffer—50 mM diethylamine, 500 mM NaCl (pHed with concentrated HCl to a final pH of 11-11.5)
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> Note about the elution buffer: The pH of 50 mM diethylamine, 500 mM NaCl is ≥ 12. I have found that the pH of this solution is too harsh on the HFE, causing more than half of it to denature. That is why I use concentrated HCl to pH the solution to pH 11-11.5.

A brief description of this step: The supernatant is run through the column. The column is then wash with the washing buffer, followed by elution of the bound protein from the column using the elution buffer. Finally, the column is re-equilibrated with washing buffer.

If I have not used the column for some time, or if the column has been used to purify HFE mutants, then I will clean the column by performing two cycles of elution buffer-washing buffer run. For each of the buffers I run about 5-10 column volumes.

1) Run 5-10 column volumes of washing buffer to equilibrate column.
2) Run about one liter of HFE-containing supernatant. I usually run the supernatant overnight using a flowrate of 0.5-0.8 ml/min, depending on how early I want to continue the purification the next day. It is a good idea to use a lower speed than the one calculated for the overnight run to minimize the possibility of running the column dry.
3) The next day the flow-rate could be increased to 1.0 ml/min.
4) Wash the column with at least 10 column volumes of washing buffer.

5) Elute the bound HFE with elution buffer (see note about elution buffer above). Collect about 30-40 fractions, 1.0-1.4 ml each, into tubes containing 100 µl of 0.1 M Tris-Cl pH 7.4. The Tris is for neutralizing the basic pH of the elution buffer.
6) Remove the fractions from the fraction collector.
7) Wash the column with at least 10 column volumes of washing buffer.
8) Read the absorbance at 280 nm (A280) for each of the fractions to determine where the eluted protein is. Before taking the A280, mix the fractions by either inverting each tube several times or by gently pipeting up and down. I use the second or third fraction to blank the spectrophotometer, since the first 5-8 fractions will correspond to the void volume of the column they should not contain protein. This is not a true blank since the buffer in the void volume will still be the washing buffer and the buffer in the fractions is the elution buffer. This, however, is not a problem since the difference in absorbance between the two buffers is negligible and the absorbance is only used for estimating the amount of protein in the fractions.
9) Pool all the fractions with an absorbance > ~0.03.
10) The amount of HFE purified could be estimated by reading the A280 of this combined sample and dividing this reading by 1.83 ml/mg to obtain the concentration expressed in mg/ml, or dividing it by 96570 M^{-1} to obtain the molar concentration.

Note: The maximum flowrate should not exceed 1.0 ml/min.

Second purification step—Gel filtration column

The buffer I use for this step is:

Tris Buffered Saline (TBS) —20 mM Tris-Cl pH 7.4, 150 mM NaCl, 0.03-0.05% Na-azide

A brief description of this step: The HFE purified using the immunoaffinity column is run through a gel filtration column to remove protein aggregates and to exchange the buffer of the sample (i.e. remove the diethylamine).

For this step I use the FPLC with the 10/25 Superdex-200 column labeled 1999, this number corresponds to the year it was bougth. Most of the time this column is equilibrated in either 20% ethanol or 0.05% Na-azide to prevent microorganisms from growing in the column. The column could be located on the FPLC, on the Biocad, or in the FPLC drawer; look for it!

FPLC setup

1) Turn on the FPLC UV detector and empty the waste.
2) Prepare and filter the TBS buffer. Place the tubing corresponding to pump A in the TBS buffer.
3) Using the "wash" command (under manual commands) replace the buffer in the FPLC lines and in pump A with the TBS buffer. This process takes about five minutes.
4) Equilibrate the Superdex-200 column with two column volumes of TBS (one column volume is 24 ml; thus, two column volumes is 48 ml). This can be done either using the manual

option or using a program. The program I use for doing this is in Bank 4 Method 5 (always double check that the method has not been changed before running it):

			COMMAND
0.00	CONC %B	0.0	start
0.00	ML/MIN	0.50	flowrate
0.00	CM/ML	0.20	chart speed
0.00	VALVE.POS	2.8	use column connected to position 8
0.00	VALVE.POS	3.8	use column connected to position 8
50.00	CONC %B	0.0	stop run

5) Normally the Superdex-200 column is located at position 8. Always check that this is the case, since there is a good chance that someone has moved the column from this position.
6) While waiting for the column to equilibrate, check that the 500-µl injection loop is connected to the FPLC. If not, remove the current loop and replace it with the 500-µl loop.
7) Manually wash the injection loop using TBS. I perform this step by aliquoting about 1.5 ml of the running buffer into a microtube, then sucking this aliquot through the injection tubing by pulling with a syringe at the other end of the injection loop. I repeat this step at least five times, to make sure that the loop and the injection tubing are clean.

Sample preparation

This step is used to concentrate the HFE purified with the immunoaffinity column. What I do most of the time is to wait until I have purified HFE from several liters of supernatant, before performing the gel filtration purification. Therefore, at this step I will have > 30 ml of HFE.

1) Concentrate the HFE solution using Ultrafree-15 concentrators. The Ultrafree-15 concentrators are used to concentrate protein samples based on a technique known as ultrafiltration. These disposable devices hold up to 15 ml of sample at a time and can be centrifuged to a maximum of ~3,000 RPM on the Sorvall table-top centrifuge (refer to the Ultrafree-15 manual for more information). To use the Ultrafree-15 just put the device in a 50-ml conical tube and fill its reservoir with up to 15 ml of the HFE sample to be concentrated. Then, centrifuge the sample at ~3,000 RPM at room temperature for about 30 minutes. This will concentrate the sample from 15 ml to about 0.5 ml.
2) Remove and save the flow-through in another tube. Add more of the HFE sample to the reservoir containing the concentrated sample.
3) Centrifuge the device at ~3,000 RPM for another 30 minutes.
4) Repeat steps 2 and 3 until all the HFE sample has been concentrated.
1) If at this time the volume of HFE is ≤ 300 µl, proceed to the next step. If it ≥ 300 µl, centrifuge again for 5-10 minutes, or more if necessary, until the volume is ≤ 300 µl.
6) There is going to be a concentration gradient in the concentrated HFE sample. Make sure to mix the sample before removing it.
7) Carefully transfer the concentrated sample to a 0.2 µm (0.45 µm if the 0.2 µm is not available) microspin filter. Rinse the Ultrafree-15 reservoir using about 100-150 µL of flow-through. Pool it with the concentrated HFE sample.

1) Centrifuge the microspin filter for five minutes at ≤ 6,000 RPM (in the microcentrifuge).
9) Check that the sample has completely flowed through the filter. If not, centrifuge for five more minutes.

Sample injection

1) Check the plotter to make sure that the trace pen is aligned with the fraction-marking pen; otherwise, the fraction marks will be off.
2) Check the fraction collector to make sure that the "fraction size" is set to 0.5 ml/fraction.
3) Check the fraction collector to make sure that the "delay" is set to 0.44 ml.
1) Set the sensitivity of the detector to an appropriate value. Usually when purifying HFE the amount of protein that is run is ≥ 2 mg; therefore, my default value for the sensitivity is 2.
5) Replace the waste container with a clean flask, to collect the sample if something goes wrong.
6) Load the 500-µl loop with the concentrated HFE sample.
7) Setup the fraction collector by putting microtubes on the collection tubes and setting the arm on the first tube.

8) Run the following program which located in Bank 4 Method 6

			COMMAND
0.00	CONC %B	0.0	start
0.00	ML/MIN	0.50	flowrate
0.00	CM/ML	0.50	chart speed
0.00	VALVE.POS	2.8	use column connected to position 8
0.00	VALVE.POS	3.8	use column connected to position 8
0.50	VALVE.POS	1.2	inject sample
2.00	VALVE.POS	1.1	stop injection
6.00	PORT.SET	6.1	start fraction collector
25.00	PORT.SET	6.0	stop fraction collector
30.00	CONC %B	0.0	stop run

9) Using the chromatogram determine which fractions contain the purified HFE.
10) Pool those fractions.
11) Determine the amount of purified protein by reading the A280 and calculating the concentration as indicated earlier. If the A280 is > 1.0, prepare ~150 µl of a 1/10 dilution of the sample and determine the concentration using this aliquot.
12) Label the tube containing the sample with your name, date, sample name, last purification step (i.e. Superdex-200), A280 reading, and calculated concentration.
13) Save the sample in the fridge (do not store sample in the freezer!).