

Purification of Histagged TfR (method by José Lebrón)

This is the procedure I use to purify histagged TfR expressed using the baculo virus expression system. It consist of three steps: (1) buffer exchange using the Amicon spiral concentrator; (2) batch/column Ni-NTA purification; (3) a gel filtration purification. Normally, the purification yield I obtain from the media received by Peter Snow is 4-10 mg/liter.

Instructions For Amicon Spiral Concentrator

Notes: (I) Filter all liquids through a 0.2 micron filter before they are used in the concentrator.
(II) Ideally you want to use a spiral concentrator with a 10 kDa cut-off. The one with a 3 kDa cut-off is too slow and the one with a 30 kDa cut-off "leaks" too much TfR through it and you will end up loosing maybe half of the sample.

- 1- Drain all the liquid from the system.
- 2- Add 500 ml of 0.1M NaOH to the chamber and let it circulate for 20 minutes.

NOTE: psi should be ~13-15, it can be adjusted with the dial in the front of the instrument.

- 3- Open the valve to drain the NaOH solution from the chamber, then close the valve.
- 4- Add 500 ml of water to the chamber and let it circulate for ~ 5 minutes.
- 5- Repeat the previous step 4-6 times to neutral pH.
- 6- Add 300-500 ml of 50 mM NaPO₄ pH 8.0 and let it circulate for 5-10 minutes, then drain the solution and close the valve.
- 7- Add the media containing the protein of interest (up to 2 liters) to the chamber
- 8- Remove the tube that is connected to the side of the spiral filter which goes to the chamber and collect the flow through into a clean container.
- 9- Concentrate the sample to ~200 ml.
- 10- Dilute sample with 50 mM NaPO₄ pH 8.0 to the original volume.
- 11- Concentrate the sample to ~200 ml.
- 12- Dilute sample with 50 mM NaPO₄ pH 8.0 to the original volume.
- 13- Concentrate sample to ~200 ml.
- 14- To collect the concentrated sample from the chamber open the valve and collect it into a clean container.
- 15- To collect the sample from the rest of the system, carefully disconnect the hose under the spiral filter and collect the liquid that will drain from the spiral filter as well as from the hose that was connected to it.
- 16- Add ~200 ml 50 mM NaPO₄ pH 8.0 to the chamber and let it circulate for 5 minutes.
- 17- Collect the rinse as described above for the sample.
- 18- Wash the concentrator with 0.1M NaOH as described above.
- 19- Add 300-500 ml of 0.2% Na azide and let it circulate for 5 minutes, then stop it and store it like that.

Batch/Column Method to Purify HisTagged Proteins Using Ni-NTA Superflow beads.

- 1- Exchange the buffer of the sample to 50 mM NaPO₄ pH 8.0 and concentrate to 200-500 ml as described above.
- 2- Once the sample buffer has been exchanged, add NaCl to 300mM, glycerol to 10% and imidazole to 10mM. Normally, the volume at this step is about 600-700 ml.
- 3- Transfer the sample to 250-ml conical bottles (orange lids), since the volume of the sample is ~700 ml I usually end up with three of them.
- 4- Take about 7.5 ml of a 50% Ni-NTA (Superflow) slurry and transfer it to a 15 ml disposable tube. For TfR purification I use about 7.5 ml of this solution for every liter of media Peter Snow give me.
- 5- Add 7 ml of 50 mM NaPO₄ pH 8.0 buffer to the beads and mix to pre-equilibrate the beads with it. Centrifuge the tube for 1-2 minutes in the clinical centrifuge at setting 2 or 3. Remove the supernatant, then add 7 ml of 50 mM NaPO₄ buffer, centrifuge and remove the supernatant as above. Finally add 7 ml of 50 mM NaPO₄ to the beads and use the resulting slurry on the next step.
- 6- Add this "pre-equilibrated" Ni-NTA beads slurry to your concentrated protein sample, equally dividing the solution among the three conical bottles.
- 7- Place the conical bottles on the shaker or rotator.
- 8- Mix for ~2 hours (longer incubations will increase background).
- 9- Spin the bottles at 3500 RPM for 5 minutes.
- 10- Remove (and save) the supernatant. I remove the supernatant by sucking it with a 3-ml syringe attached to a tube which is connected to a vacuum tramp
- 11- Transfer the Ni-NTA beads to a column support.
- 12- Wash the beads with 10-20 column volumes of 300 mM NaCl, 50 mM NaPO₄ pH 8.0, 10 mM imimidazole (Buffer A).

Note: It is a good idea to pH the imimidazole stock solution to pH 8.0, otherwise its pH is more alkaline than the NaPO₄ solution and could affect the final pH of your solutions.

- 13- Wash the beads with 10-20 column volumes of 300 mM NaCl, 50 mM NaPO₄ pH 8.0, 20 mM imimidazole (Buffer B; i.e. Buffer A with 10 mM Imidazole instead).
- 14- Elute bound protein with 10 column volumes of 300 mM NaCl, 50 mM NaPO₄ pH 8.0, 250 mM imimidazole (Elution buffer; i.e. Buffer B with 250 mM Imidazole instead).
- 15- The amount of TfR purified could be estimated by reading the A₂₈₀ of this sample and dividing this reading by 1.293 ml/mg to obtain the concentration expressed in mg/ml, or dividing it by 93790 M⁻¹ to obtain the molar concentration. The molar concentration will correspond to the concentration of TfR monomers. Multiply 187580 M⁻¹ to obtain the molar concentration of the TfR homodimer.

Note: Use the elution buffer to blank the spectrophotometer.

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 histagged TfR obtained using the Ni-NTA beads is run through a gel filtration column to remove protein aggregates and to exchange the buffer of the sample (i.e. remove the imidazole).

For this step I use the FPLC with the 10/25 Superdex-200 column labeled 1999, this number corresponds to the year it was bought. 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 located in Bank 4 Method 5 (before running it always double check that the method has not been changed):

| | | | <u>COMMAND</u> |
|-------|-----------|------|------------------------------------|
| 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- μ l injection loop is connected to the FPLC. If not, remove the current loop and replace it with the 500- μ l 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 TfR purified with the Ni-NTA beads. Normally the volume of the TfR eluted from the Ni-NTA column is 50-100 ml.

- 1) Concentrate the TfR 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 TfR 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 TfR sample has been concentrated.

- 1) If at this time the volume of TfR is 300 μ l, proceed to the next step. If it is 300 μ l, centrifuge again for 5-10 minutes, or more if necessary, until the volume is 300 μ l.
- 6) There is going to be a concentration gradient in the concentrated TfR sample. Make sure to mix the sample before removing it.
- 7) Carefully transfer the concentrated sample to a 0.2 μ m (0.45 μ m if the 0.2 μ m is not available) microspin filter. Rinse the Ultrafree-15 reservoir using about 100-150 μ L of flow-through. Pool it with the concentrated TfR 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 TfR 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- μ l loop with the concentrated TfR sample.
- 7) Inject the sample onto the Superdex-200 column, which should be already equilibrated with the TBS buffer, using a flow rate 0.5 ml/min.
- 8) Run the corresponding program. I like to use the program located in Bank 4 Method 6 (always double check that the method has not been changed before running it):

| | | | <u>COMMAND</u> |
|-------|-----------|------|------------------------------------|
| 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) Determine which fractions contain the purified TfR sample.
- 10) Pool those fractions.
- 11) Determine the amount of purified protein by reading the A280 and calculating the concentration as indicated above. If the A280 is > 1.0, prepare ~150 μ l 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!).