

Purification of TRX-Exon1Htt39Q-His

Reserve Incubator ahead of time for two days. Overnight for the first day and all day for the second

Day1:

1. Make 6*1L and 2*250ml LB media and autoclave in the morning
2. In the evening, add 250ul of 100mg/ml Ampicillin stock to each of the 250ml flask
3. Add 1ml of 100mg/ml ampicillin stock to each of the 1L flasks
4. Inoculate just 1 of the 250ml flask with Htt39Q glycerol stock using a pipette tip. This is the overnight culture that will be used the next day.
5. Place it in the 37°C incubator and shake it overnight (about 16 hrs)

Day2:

1. Stop the shaker and spin down the o/n culture in a large centrifuge bottle. Use the centrifuge with the "for bacteria use" label on it. Spin for about 5-10 mins at 3000 rpm. Take out the bottle and discard just the supernatant (broth) into a bucket that is in the bug room (this bucket should have a little bit of bleach in it).
2. To the pellet add 75mls. of LB broth (this is from the second 250ml of LB you made yesterday) This is called the refresher stock. Carefully suspend the pellet using a pipette. DO NOT vortex.
3. After resuspension, add about 12mls of this culture to each of the 1L flasks with LB.
4. Let them shake. Check the O.D. at 600nm periodically.
5. In the meantime prepare IPTG stock. Weigh out 1.55 g of IPTG and suspend it in 6.5ml of milliQ water. Vortex to get all the IPTG in solution. This is a 1M stock.
6. When the O.D is between 0.4-0.6 it's time to induce the cells with IPTG. For Htt39Q, it takes about 2.5 hours to reach this O.D. Before inducing, save a 3ml sample of the culture. This is the "- IPTG" sample.
7. To induce the culture add 1ml of the IPTG stock to each of the 1L culture flasks. Final concentration is 1mM IPTG in 1L of culture.
8. Continue shaking for another 3 ½ - 4 hours at 30°C. This time might vary depending on what construct you are expressing. For htt39Q, between 3-4 hrs you get maximum expression of protein.
9. Harvest the culture in this time range by centrifuging them for 20 mins. Discard the supernatant in the bacterial waste container carefully. Save the pellets for future use. Label the containers and store them in -20 freezer.
10. Clean up all the flasks with bleach and discard the bacterial waste container and rinse it. Add little bleach to the container for next use.

Day 3 (or later):

1. Buffer Recipe:
 - 8x Binding Buffer (8x = 40 mM imidazole, 4 M NaCl, 160 mM Tris-Hcl pH 8)
 - 8x Wash Buffer (8x = 480 mM imidazole, 4 M NaCl, 160 mM Tris-Hcl pH 8)
 - 4x Elute Buffer (4x = 4 M imidazole, 2 M NaCl, 80 mM Tris-Hcl pH 8)
 - 4x Strip Buffer (4x = 400 mM EDTA, 2 M NaCl, 80 mM Tris-Hcl pH 8)
 - 8x Charge buffer (8x = 400 mM NiSO₄)
2. To charge column, do the following:
 - 3 volumes ddH₂O
 - 5 volumes 1x charge buffer
 - 3 volumes 1x binding buffer
3. Note: the His bead can be used 2 times. After the first purification, run 2 bed volumes of ddH₂O, then 4 volumes of strip buffer. Then charge the column as step # 2.
4. In the morning (or day before) wash, charge and equilibrate His-bind resin (GE Healthcare)
5. Take out the frozen pellets from day before and let them come to room temperature. Keep them on ice or room temp for a short time to thaw out.
6. After they are sort of thawed out, add about 10 mls of 1X binding buffer (used also for his-bind column equilibration). Resuspend the pellet in binding buffer. Can also be vortexed until the pellet is

completely dissolved. Repeat this for the other bottles. In the end rinse out all bottles with little bit more of the binding buffer. Typical volume for 3L of resuspended pellet is about 50-60 mls. Keep all of this in a small beaker in an ice bucket. Divide the 6L into 2x3L, i.e. 2x 50 ml, sonicate separately, this will yield better bacterial lysis.

7. Place this beaker on ice inside the sonicator. Use the large sonication tip. The Duty Cycle needs to be at "constant" and the Output Control should be at 3. Sonicate for 1-2 mins max and let cool for few mins. This should be repeated until the lysate is clearer. For Htt39Q, this will usually take 4-5 rounds of sonication.
8. After sonication, pour the lysate in small centrifuge tubes and spin them for about 20 mins at 17000 rpm.

Day 3 : His-Bind Column (Use Biorad empty column):

1. Pour off the supernatant carefully into a tube/beaker. This will be the start material for the His-bind column. Generally it's good to save the pellet until you are sure that the protein is in the supernatant.
2. Once the column is packed, washed, charged and equilibrated connect it to the fraction collector. Set the flow rate to about 2ml/min; chart speed at 0.5 mm/min; Red pin is at 20mV ; Blue pin is at 50mV. Collect 70 fractions with about 10mls per fraction. You can stop collecting fractions if you are sure all the protein is eluted. During my runs, this was around fraction #55-60.
3. Start loading the supernatant and keep an eye on it to make sure the tubing at the top does not pop off due to high pressure.
4. After all the lysate is loaded (save about 50ul to run a gel later), start washing the column with 1X binding buffer.
5. Once the chart recorder pin starts to come back to baseline from the peak, start pumping 1X wash buffer.
6. Again when this peak comes to baseline pump the elute buffer.
7. Check to make sure where the protein is eluting off by running SDS PAGE. I run 25ul of sample + 25ul of load buffer with BME. Initially to get an idea, I would run the start material, couple of the wash fractions and the elute fractions. Usually you see very little huntingtin in the wash fractions.
8. Pool the eluted fractions according to the amount of protein present in them.
9. Dialyze these pools overnight in 10mM Tris, pH 7.4 with 0.25mM EDTA. DO NOT ever leave the eluted fractions in the Imidazole elute buffer because they will precipitate out of solution.
10. Day 4: Change the dialysis buffer at least 2 more times (for a total of 3 x 4 L changes).

Day 5: DEAE HPLC Column (Sigma):

Book the biocad HPLC system few days in advance. There is a sign up sheet right above the biocad.

1. Carefully collect the dialyzed Htt39Q from the dialysis bags/cassettes.
2. Filter the samples with 0.22 μ m syringe filter. If there is visible precipitate, spin the sample for 5 minutes at 10000 rpm. Do not skip this step, because unfiltered sample will clog the DEAE column.
3. Connect the DEAE 1PW (small column) to the HPLC system. Wash it with buffer A for a couple of bed volumes. The max flow rate for this column is 1ml/min. For the bigger DEAE 5pw column, the flow rate is 5ml/min.
4. Run the saved program under method/Kartika/httdeae3. The gradient buffers used are:
Buffer A – 10mM Tris pH 7.4; 0.25mM EDTA
Buffer B – 10mM Tris pH 7.4; 0.25mM EDTA; 0.5M NaCl
To make these buffers I used the Weisgraber lab's 1M Tris pH 7.4 stock bought from a company. I also used the 500mM EDTA stock.

Note: for 6 L culture bacteria divide the purified His eluted protein into 2 runs.

The following are the program used for biocad for TrxHtt39Q:

- 2 column volume of buffer A
- inject sample 4 x 3000 ul each injection (use 5 ml sample loop)
- 1 column volume of buffer A
- in 3 column volume, set gradient to 20% buffer B
- in 1 column volume, set gradient to 20% buffer B
- in 6 column volume, set gradient to 100 % buffer B
- in 4 column volume, keep the gradient at 100% buffer B

- in 5 column volume, set gradient to 100% buffer A
5. Run SDS-Page of the fraction, pool the pure fraction, and concentrate with centrifugation.
 6. Dialyze the salt off into 5 mM Tris pH 8 buffer. Flash freeze the TrxHtt39Q in aliquots and store them at -80°C . This purified Htt will be used to make co-complex with 3B5H10 Fab.

Note:

1. Store all the columns with binding buffer containing 0.05% Na azide.
2. Flash frozen TrxHtt39Q that is stored at -80°C , has been observed to make needles when it is complexed with 3B5H10 Fab.