Standard operating procedure (SOP)

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Title: **Production and purification of Huntingtin (Htt) proteins expressed by mammalian cells**

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1. Purpose

Production of normal FL-Htt17 (Htt protein with 17 Q) and mutant FL-Htt46 (Htt protein with 46 Q) in adherent mammalian cell lines B1.21.03 and C2.6.02 and subsequent purification of the proteins.

2. Materials

2.1. Cells

- The cell line B1.21.03 expresses Htt17 after induction by doxycycline.
- The cell line C2.6.02 expresses Htt46 after induction by doxycycline.

Both cell lines are HEK293 cell based, contain the Tet-on inducible expression system and express Htt17 and Htt46, respectively, after induction with doxcycylin.

2.2. Equipment

- General cell culture equipment (sterile work bench; incubator 37°C, 5%CO_2, 95% humidity)
- Table-top centrifuge (Heraeus, Multifuge 3 S-R)
- End-over-end shaker (Snijders, Test-tube rotors, 34528)
- High-speed centrifuge (Sorvall RC 6 Plus with Rotor F21s-8x50y)
- High-speed tabletop centrifuge (Eppendorf 5417R)
- AKTA purifier FPLC system (GE Healthcare).

2.3. Reagents and materials

2.3.1 For cell cultivation, induction and harvest

- MEM alpha (Invitrogen , 61100-087)
- FBS (Gibco, 10270)
- 100x Penicillin-streptomycin-glutamine (Gibco, 10378) or 100x Glutamax (Gibco, 35050-038)
- G418, 50mg/ml (Gibco, 10131)
- Hygromycin B, 50mg/ml (Clontech, 631309)
- Doxycycline, 1mg/ml in water, sterilie filtered with 0.2µm filter and stored at -20 °C.
- Trypsin-EDTA 0.05% (Gibco, 25300)
- 0.5M EDTA (Applichem, A3553) in water, pH adjusted to 8.0 with NaOH.
- Complete EDTA-free Proteinase inhibitor (Roche, 0469313200)
- PBS (PAA, H15-002)

2.3.2 For protein purification

- Anti-Flag M2 beads (Sigma, A2220)
- 20ml chromotograpy columns (Bio-Rad, 732-1010)
- 20% Tween 20 (Roth, 9127), stored at -20 °C.
- Flag-M2 peptide (Sigma, F4799), dissolved in TBS and stored in aliquots at -20 °C.
- Superose 6 10/300 GL column (GE Healthcare, 17-5172-01)
- BCA Protein Assay Kit (Pierce, 23235)
- Ultra-4 Centrifugal Filter Units, 30kDa (Amicon, UF803024)
- 1M DTT (Applichem, A1101) in water, stored at -20 °C.
- 10% Chaps (Applichem, A1099) in water, stored at -20 °C
- 1M Tris (USB, 77-86-1) pH 8.0: dissolve in water and adjust pH to 8.0 with HCl
- NaCl (Sigma, 31434)
- KCl (Applichem, A2939)
- MgCl (Applichem, A3618)
- Glycerol (Applichem, A2926)
- ATP (Sigma, A3377)
- Glycine (Sigma, 33226)
- NaOH (Applichem, A1551)

2.4. Media and buffers (in order of use)

Cell culture medium: Alpha-MEM, 10% FBS, 1% Pennicillin-streptomycin-glutamine, 100ug/ml G418, 37.5µg/ml Hygromycin B

Cell culture medium for induction: Alpha-MEM, 10% FBS, 1x Pennicillin-streptomysin-glutamine, 100ug/ml G418, 37.5µg/ml Hygromycin B, 1µg/ml doxycycline.

Cell detachment buffer: PBS with 20mM EDTA

Buffer A: 50mM Tris, 500mM NaCl, 5% glycerol, pH 8.0.

TBS: 50mM Tris, 150mM NaCl, pH8.0.

Glycine HCl with 150mM NaCl: 100mM glycine, 150mM NaCl, adjust pH to 3.5 with HCl

Buffer B: 50mM Tris, 500mM KCl, 5mM $MgCl_2$, 5% glycerol, pH 8.0

Buffer C: 20mM Tris, 200mM KCl, 5mM $MgCl_2$5% glycerol, pH 8.0

100mM ATP: dissolved in H2O and stored at -20 °C.

Flag elution buffer: Buffer A with 200µg/ml Flag-M2 peptide

TBS with 50% glycerol: 50mM Tris, 150mM NaCl, 50% glycerol, pH 8.0

GFC buffer: 20mM Tris, 500mM NaCl, 0.4% Chaps, 5mM DTT and 5% glycerol. Filtered with 0.2µm filter and stored at 4 ^{o}C.

3. Procedure

3.1. Handling and cultivation of cell lines

3.1.1 Thawing of frozen cells

Keep frozen cells in a liquid nitrogen tank (gaseous phase) for long-term storage.
To start a new culture, prepare a culture dish or flask with culture medium, then thaw cryovial quickly in a 37°C water bath. Add thawed cells to the prepared culture vessel and change medium on the following day to remove DMSO (Alternatively, cells can be centrifuged directly after thawing and resuspended in fresh medium).

3.1.2 Maintenance of culture

Split cells every 3-4 days by a factor of 8-10.
Wash cells with PBS and detach with trypsin (incubation time ~3 min at 37°C). At a seeding density of $2x10^4$ cells/cm^2, both cell lines generally reach a confluency of 70-90% ($2.5\text{-}3x10^7$ cells per 15cm dish) after 3-4 days.

3.1.3 Freezing of cell stock

Detach cells at 70-90% confluency. Centrifuge at 300g for 2 min and resuspend pellet in freezing medium (FBS, 10% DMSO) at $2\text{-}5x10^6$ cells/ml. Fill cell suspension in cryovials and place vials in freezing device with coolant isopropanol. Transfer to liquid nitrogen tank after 10-24h.

3.2. Seeding of cells and induction

1. split 15cm dishes at 80% confluence at ratio 1:8 (e.g. 10 dishes to 80 dishes).
2. After 3 days, exchange the culture medium with the same volume of induction medium (containing 1µg/ml doxycyclin) to induce Htt expression.
3. After three days after induction, check cells for GFP expression under fluorescence microscope.
   In B1.21.03 and C2.6.02 cells, both a Htt cDNA and a EGFP cDNA are controlled by the same Tet inducible promoter. Therefore, after induction EGFP will be co-expressed with htt proteins, which can be monitored under a fluorescence microscope as a marker of htt expression. In general, over 80% of induced cells should show visible GFP expression.

4. Aspirate medium and replace it with 10ml cell detaching buffer.
5. Gently swirl the dish to detach cells, about 3-5 minutes.
6. Fully detach cells by pipetting up and down.
7. Collect and harvest cells by centrifugation at 400g for 10 min.
8. Wash cells twice with PBS and centrifuge at 400g for 10 min.
9. Suspend cells in 20ml buffer A with 5mM EDTA and 1x poteinase inhibitor. Always keep cells on ice (long-term storage possible at -80 ^{o}C).

3.3. Purification of Htt with anti-flag affinity resin

1. Lyse cells by three consecutive freeze and thaw cycles: freeze in liquid nitrogen and thraw at 37 ^{o}C in a water bath.
2. Centrifuge cell lysate at 16,000 rpm (about 30,000g) for 3 hours at 4 ^{o}C to pellet cellular debris and DNA.
3. After centrifugation, take the supernatant and further filter through 0,2µm filter.
4. Prepare Flag affinity columns during the centrifugation (1 column per 40 harvested 15cm dishes).
    a) Place an empty chromatography column on a firm support.
    b) Thoroughly suspend the anti-flag M2 resin and transfer 1.4 ml resin into column
    c) Remove the bottom cap and allow the gel bed to drain
    d) Wash the column with 20ml TBS
    e) Wash the column with 3ml 0,1M glycine HCl with 150mM NaCl (pH3.5) to elute any possible protein binding to Flag resin. Do not leave the column in glycine HCl for longer than 20 min
    f) Immediately wash the column with 20 ml TBS to equilibrate the resin for use.
    g) Do not let the resin dry!

5. Add the cleared cell lysate into the Flag affinity column and incubate the anti-Flag affinity beads and cell lysate with gentle mixing on an end-over-end shaker for 2 hours at 4 ^{o}C.
6. After incubation, allow the cell lysate to flow out thoroughly.
7. Wash the column with 15 ml buffer A with 0.01% Tween20 under gentle agitation on an end-over-end shaker for 15 min at 4 ^{o}C.
8. Wash the column with 15 ml buffer B with 0.01% Tween20 under gentle agitation on an end-over-end shaker for 15 min at 4 ^{o}C.
9. Wash the column with 10 ml buffer C with 5mM ATP and 0.01% Tween20 under gentle agitation on an end-over-end shaker for 30 min at RT.
10. Wash the column with 15 ml buffer B with 0.01% Tween20 under gentle agitation on an end-over-end shaker for 10 min at 4 ^{o}C.
11. Wash the column with 15 ml buffer A with 0.01% Tween20 under gentle agitation on an end-over-end shaker for 10 min at 4 ^{o}C.

12. Elute Htt protein from the Flag-affinity resin with 3ml elution buffer and collect the Htt protein. Add the elution buffer gently into column and let it flow through beads slowly by gravity. Elute beads four times with this 3ml eluate to elute as much Htt as possible from the Flag-affinity column.
13. After elution, immediately wash the Flag-affinity column with 3ml 0,1M glycine HCl with 150mM NaCl (pH3.5) to elute any protein still binding to the column. Then wash the Flag-affinity column with 20ml TBS followed by 10 ml TBS with 50% glycerol and store it at -20 ^{o}C. The Flag-affinity column can be reused for at least 5 times with slightly reduced binding capacity.

3.4. Further purification with superose-6 GFC

1. Connect superose 6 10/300 GL column to AKTA purifier with a 1ml sample loop.
   In general, the column is stored in 20% ethanol. Connect column to FPLC system with drop-to-drop method to prevent introducing air into the system.
2. Wash the column with 2 column volumes (CV, about 48 ml) PBS.
   Flow rate: 0.6 ml/min.
3. Equilibrate the column with 2CV GFC buffer (Flow rate: 0.6 ml/min).
4. Measure the protein concentration of the Flag-purified Htt, using the BCA Kit, and concentrate it to about 500µg-1mg/ml by using Amicon ultra 30kDa filter device.
5. Add DTT to the Flag-purified Htt to 5mM and clear it by centrifugation at 14,000 rpm using an Eppendorf table centrifuge, before loading the sample into FPLC.
6. Inject 600µl Htt protein (concentrated and cleared by centrifugation) into the 1ml sample loop of FPLC and run the program (Htt purification) to further purify Flag-purified Htt.

   <u>Major parameters of the Htt purification program:</u>
   Flow rate: 0.6ml/min. pressure limit: 1.5 MPa. Equilibrate the column with 0.1CV GFC buffer, empty sample loop with 5ml buffer, elute the protein with 1CV GFC buffer (about 24ml) and collect protein with 1ml/tube.

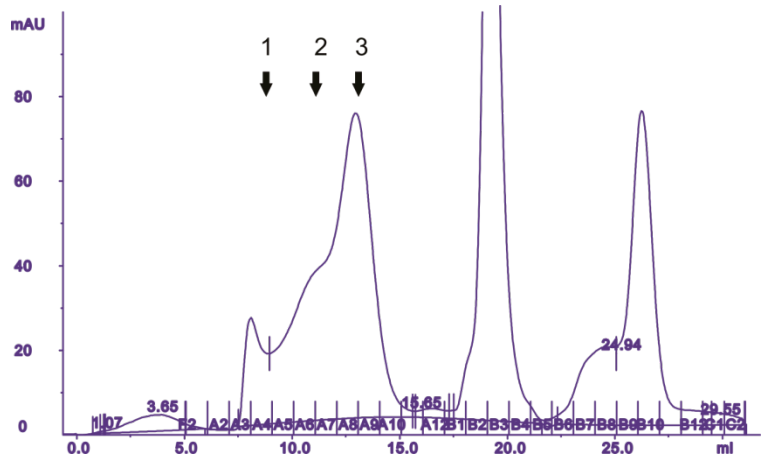

Fig. 1. Typical elution pattern of Htt from Superose 6 GFC. Htt elutes mainly in 3 peaks (1, 2 and 3). Peak 3 is composed of monomeric Htt protein.

7. Combine all fractions of peak 3.
8. After measuring the protein concentration as above, concentrate Htt protein to about 200-300µg/ml by using Amicon ultra 30kDa filter device.
   Protein should be frozen in liquid nitrogen and stored in aliquots at -80 ^{o}C.
9. Wash superose 6 column with 2CV PBS followed by 2CV 20% ethanol and store it at 4 ^{o}C.

Note:

Expected protein yield: The induction of ~4x10^{8} cells (40x15cm culture dishes) generally yields 500 µg-1mg Htt protein after Flag purification and 250 µg-500 µg Htt protein after further purification by GFC.