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Efficient and Scalable Production of Full-length Human Huntingtin Variants in Mammalian Cells Using A Transient Expression System
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1 **TITLE:**
2 Efficient and Scalable Production of Full-length Human Huntingtin Variants in Mammalian Cells
3 Using A Transient Expression System
4
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23 **SUMMARY:**
24 We provide scalable protocols covering construct design, transient transfection, and expression
25 and purification of full-length human huntingtin protein variants in HEK293 cells.
26
27 **ABSTRACT:**
28 Full-length huntingtin (FL HTT) is a large (aa 1–3,144), ubiquitously expressed, polyglutamine
29 (polyQ)-containing protein with a mass of approximately 350 kDa. While the cellular function of
30 FL HTT is not entirely understood, a mutant expansion of the polyQ tract above ~36 repeats is
31 associated with Huntington's disease (HD), with the polyQ length correlating roughly with the
32 age of onset. To better understand the effect of structure on the function of mutant HTT (mHTT),
33 large quantities of the protein are required. Submilligram production of FL HTT in mammalian
34 cells was achieved using doxycycline-inducible stable cell line expression. However, protein
35 production from stable cell lines has limitations that can be overcome with transient transfection
36 methods.
37
38 This paper presents a robust method for low-milligram quantity production of FL HTT and its
39 variants from codon-optimized plasmids by transient transfection using polyethylenimine (PEI).
40 The method is scalable (>10 mg) and consistently yields 1–2 mg/L of cell culture of highly purified
41 FL HTT. Consistent with previous reports, the purified solution state of FL HTT was found to be
42 highly dynamic; the protein has a propensity to form dimers and high-order oligomers. A key to
43 slowing oligomer formation is working quickly to isolate the monomeric fractions from the
44 dimeric and high-order oligomeric fractions during size exclusion chromatography.

45

Size exclusion chromatography with multiangle light scattering (SEC-MALS) was used to analyze the dimer and higher-order oligomeric content of purified HTT. No correlation was observed between FL HTT polyQ length (Q23, Q48, and Q73) and oligomer content. The exon1-deleted construct (aa 91–3,144) showed comparable oligomerization propensity to FL HTT (aa 1–3,144). Production, purification, and characterization methods by SEC/MALS-refractive index (RI), sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), western blot, Native PAGE, and Blue Native PAGE are described herein.

53

INTRODUCTION:
Huntington's disease (HD) is a rare neurodegenerative disease primarily characterized by unsteady and involuntary motor movement, as well as cognitive and psychiatric alterations, such as personality changes and apathy[1,2]. HD is associated with an expansion of the CAG repeat tract located in exon 1 of the huntingtin gene (*HTT*) to more than 35 repeats, with a higher number of CAG repeats correlating with an earlier onset of the disease[3,4]. The translational product of *HTT*, the huntingtin protein (HTT), is implicated in neuronal viability and brain development[5–9].

61

HTT is a scaffolding protein reported to participate in a wide range of cellular processes, vesicle transport, cell division, ciliogenesis, and autophagy[10,11]. However, the molecular pathogenesis of HD is not entirely clear, and the identification of key protein interactors mediating the pathological impact of polyQ-expanded mHTT is lacking. Some research suggests a gain of toxic function from mHTT driven by the oligomerization propensity of the expanded HTT protein, as HTT aggregates have been identified in neurons and glia in HD patients and animal models of the disease[12–17]. To fuel the investigation of the function and structure of FL HTT and mHTT variants and supply researchers with high-quality protein standards for assay development, a robust and scalable supply of homogenous recombinant protein is needed.

71

Due to its size (aa 1–3,144, numbering based on polyQ length Q23), proteolytic instability, and propensity to aggregate, FL HTT has proven difficult to express and isolate as a soluble protein. Previously, the exon 1 region (aa 2–90) of HTT has been expressed and purified at a large scale using various tags that can increase the solubility of the protein in *Escherichia coli*[18–20]. FL HTT was first expressed and purified in an insect cell expression system using baculovirus[21,22], and low-resolution 30 Å electron microscopy (EM) structures of chemically crosslinked FL Q23-HTT and Q78-HTT were reported[23]. The investigation of HTT structure was further advanced when the production of FL Q17, Q46, and Q128-HTT with native posttranslational modifications (PTMs) was achieved in human cells using stable cell lines or adenovirus expression systems[24]. These studies suggest that although purified HTT mainly exists in the monomeric state, it also tends to form high-order oligomers and aggregates.

83

Analytical ultracentrifugation of FL Q128-HTT, with a highly expanded polyQ region, afforded more oligomeric and aggregate fractions than the protein with the non-expanded polyQ region[24]. Using a stable cell line, a strategy has been successfully adapted to stabilize FL HTT by co-expression with the interaction partner HAP40. A cryo-EM structure of the FL HTT and HAP40 complex has been solved at an average 4 Å resolution using the purified protein complex

89   (PDB:6EZ8)[25]. This co-expression strategy has been adapted successfully to a baculovirus system,
90   and a series of high-quality HTT variants with different polyQ lengths have been expressed and
91   purified from insect cells[26]. Since then, more cryo-EM structures of the complex of HTT with
92   variable polyQ lengths and HAP40 and higher resolution structures were solved and deposited in
93   the Protein Data Base[27,28] (PDB: 7DXK, 7DXH, 6X9O).
94
95   We optimized a transfection and expression method in HEK293 cells, using polyethylenimine
96   (PEI), for rapid transient expression of FL HTT. As a proof-of-principle, FL HTT variants containing
97   23 glutamines (FL Q23-HTT) were first purified and characterized using a modification of a
98   purification method described previously[24]. This transient transfection method is convenient,
99   highly efficient, and scalable; it can produce purified HTT with yields of 1–2 mg/L, comparable to
100 the stable cell line method reported[24]. Because the protein is produced in a human cell line, the
101 HTT produced is more likely to have native human PTMs when subjected to mass spectrometry
102 proteomics analysis[11,29–31]. Milligram quantities of the FL Q48-HTT, FL Q73-HTT, and exon1-
103 deleted (ΔExon1-HTT) variants of FL HTT were produced, demonstrating that the transient
104 expression method is especially useful in rapidly producing alternative variants of HTT without
105 depending on the time-consuming effort required to establish stable cell lines for production.
106
107 The following protocol exemplifies the standard method used in these authors' laboratory for
108 cell culture, transfection, protein purification, and postpurification protein characterization to
109 produce FL Q23-HTT from a 2 L cell culture. The protocol can be scaled up to larger cultures or
110 adapted to purify other HTT variants. Up to 10 L cell cultures of FL HTT and various site or
111 truncation mutations of HTT and HTT homologs have been performed successfully in the
112 laboratory using the same protocol. Purified FL HTT contains a high percentage of monomers
113 along with dimers and higher-order oligomers. The same aggregate profile is observed among
114 the variants produced (Q23, Q48, Q73, and ΔExon1). As aggregation can occur when proper care
115 is not taken, a formulation and freeze–thaw stability study was conducted to identify the best
116 conditions for protein handling. Methods, such as Blue Native PAGE and SEC/MALS-RI, are also
117 described to analyze the HTT oligomer content as part of the quality control process. To benefit
118 the HD research community, plasmids and HTT proteins described in this study are also deposited
119 in the HD Community Repository at the Coriell Institute (www.coriell.org/1/CHDI).
120
121 **PROTOCOL:**
122
123 **1.   Design and production of constructs for FLAG-tagged *HTT* mammalian expression**
124
125 1.1.   Retrieve the full-length human HTT protein sequence (P42858) from National Center for
126 Biotechnology Information (https://www.ncbi.nlm.nih.gov/).
127
128 NOTE: Researchers need to be familiar with HTT's domain organizations and maintain HTT core
129 3D structure when designing constructs for HTT mutants.
130

131   1.2.   Request a gene synthesis service to perform codon optimization for human cell
132   expression based on the sequence of P42858. Change the polyQ number from Q16 to the desired
133   Q length (Q23 was chosen as the first construct here) and synthesize the full-length *HTT* gene.
134
135   NOTE: The synthesized codon-optimized full-length Q23-HTT construct was delivered as an insert
136   in the pUC18 plasmid in this study.
137
138   1.3.   Optional: Add features to facilitate the cloning of different Q lengths and purification in
139   the constructs.
140
141   NOTE: A tobacco etch virus (TEV) cleavage site and FLAG purification tag
142   (AAAENLYFQGDYKDDDDK) were added to the C-terminal end of the constructs. Two HindIII sites
143   were designed in the constructs to encompass the polyQ region (translated protein sequence is
144   not changed by introducing HindIII sites). This allows the researcher to change HTT's Q length by
145   restriction enzyme digestion and ligation without resynthesizing the full *HTT* gene.
146
147   **2.   Clone the synthesized HTT constructs into pcDNA3.1.**
148
149   2.1.   Digest 5 µg of pUC18-Q23-*HTT* and 5 µg of pcDNA3.1 using 2 µL of NheI and PmeI each at
150   37 °C for 2 h.
151
152   2.2.   Run a 0.5% w/v agarose gel and purify the Q23-*HTT* fragment and the digested pcDNA3.1
153   vector using an agarose gel extraction kit. Quantitate the concentrations of purified DNA by OD_{280}
154   using a UV spectrometer that can measure microliters of samples.
155
156   NOTE: $OD_{260/280}$ ranging from 1.8 to 2.0 is typically observed. The synthesized FL HTT is supplied
157   as an insert with NheI and PmeI at both ends in a pUC18 plasmid. Use other restriction enzymes
158   if *HTT* is synthesized differently.
159
160   2.3.   Use 10 ng of digested pcDNA3.1 vector in the reaction. Ligate the purified DNAs at 1:1
161   (HTT:pcDNA3.1) molar ratio in a 10 µL reaction at room temperature for 5 min using T4 DNA
162   ligase.
163
164   2.4.   Transform the ligated product into competent *E. coli* cells (see the **Table of Materials**)
165   using the protocol specified by the ligase manufacturer.
166
167   2.5.   Pick up 6 single colonies and make overnight cultures in 4–6 mL of LB supplemented with
168   100 µg/mL carbenicillin at 37 °C.
169
170   2.6.   Allocate 1 mL from each overnight culture. Add glycerol to 25% and save the glycerol stock
171   at -80 °C. Purify the remaining overnight culture using a mini-prep kit according to the steps
172   specified in the user's manual.
173

174   2.7.   Sequence all plasmids using sequencing primers spanning across the transcription region
175   of the plasmid. Choose one glycerol stock that has the correct sequence as the master glycerol
176   stock and discard the rest.
177

178   2.8.   Optional: Request a gene synthesis service to synthesize DNA with the different Q lengths
179   (Q48, Q73, and ∆Exon1) that span the two HindIII sites in the pcDNA3.1-Q23-HTT plasmid. Digest
180   pcDNA3.1-Q23-HTT and the newly synthesized DNAs using HindIII, and religate them with T4
181   ligase as in steps 2.2–2.7 to make FL HTT with different polyQ lengths in the pcDNA3.1 plasmid.
182

183   NOTE: Plasmid constructs used in this study are also available directly from the HD Community
184   Repository at the Coriell Institute (www.coriell.org/1/CHDI); see the **Table of Materials**.
185

186   **3.**   **GIGA prep endotoxin-free plasmid DNA for large scale transfection**
187

188   3.1.   Streak the bacterial glycerol stocks of pcDNA3.1-Q23-*HTT*-TEV-FLAG on an LB agar plate
189   with carbenicillin (100 µg/mL). Incubate the plate at 37 °C for 16–24 h until single colonies appear.
190

191   3.2.   Pick up a single colony, inoculate a 5 mL starter culture in a rich medium formulated for
192   plasmid amplification with carbenicillin (100 µg/mL), and grow at 37 °C for 8 h.
193

194   3.3.   Choose an endotoxin-free GIGA plasmid purification kit. Follow the steps outlined in the
195   manual of the plasmid GIGA kit to purify the pcDNA3.1-Q23-HTT-TEV-FLAG plasmid.
196

197   3.4.   Measure the plasmid endotoxin levels using a limulus amoebocyte lysate (LAL)-based
198   endotoxin quantification kit. Follow the procedure specified in the manufacturer's manual.
199

200   NOTE: A high-quality, low-endotoxin level plasmid purification is essential to obtain good
201   transfection efficiency. Using this protocol, 20–40 mg of plasmid (supercoiled form >80%) can be
202   obtained per L of bacterial culture at plasmid concentrations > 4 mg/mL. A properly purified
203   plasmid should have an endotoxin level < 30 EU/mg. $OD_{260/280}$ ranging from 1.8 to 2.0 is typically
204   observed.
205

206   **4.**   **Large-scale transfection of 2 L of HEK293 cells by polyethyleneimine (PEI)**
207

208   4.1.   Add 1 g of PEI 25K to 1 L of endotoxin-free water with stirring. Adjust the pH to 2.0 using
209   100 mM HCl and stir until all the PEI 25K dissolves. Adjust the pH to 7.0 using 100 mM NaOH
210   solution and filter through a 0.2 µm filter. Aliquot and store at -20 °C for up to a year.
211

212   NOTE: Aliquots of PEI can be kept at 4 °C for up to two weeks but should never be refrozen after
213   thawing.
214

215   4.2.   Propagate HEK293 cells in the growth medium (see the **Table of Materials**) supplemented
216   with penicillin-streptomycin (final concentration at 5 U/mL for penicillin and 5 µg/mL for
217   streptomycin) in a humidified shaker incubator at 37 °C, 90 rpm, 5% CO_2 for 18–24 h. Dilute the

cells to 2 L at a density of ~1.2×10^6 cells/mL using the growth medium in 5 L Erlenmeyer flasks one day prior to transfection.

4.3.   Continue to grow the cells at 37 °C, 90 rpm, 5% CO_2 for 18–24 h. Measure the cell parameters using an auto cell counter capable of measuring cell density and viability following the user's manual.

NOTE: The cell density should double, and viability should be >95%. The cell density before the transfection should be approximately 2.0×10^6–2.4×10^6 cells/mL. Dilute the cells to the desired density before transfection when necessary.

4.4.   Calculate the amounts of plasmid and PEI required for the transfection; use 1 mg of plasmid and 3 mg of PEI for transfection of each liter of cell culture. Allocate 2 mg of plasmid and 6 mg of PEI needed for a 2 L transfection.

4.5.   Dilute the plasmid and PEI individually into a volume of phosphate-buffered saline equal to 1/20th of the total volume of cell culture (100 mL each for a 2 L transfection) and incubate at room temperature for 5 min. Mix the diluted plasmid and PEI by gentle swirling and incubate the mixture at room temperature for 30 min.

NOTE: The mixture will appear slightly cloudy after incubation.

4.6.   Add the mixture to the cell culture and swirl gently to mix them.

4.7.   Grow the cells at 37 °C, 5% CO_2, 90 rpm for 24 h.

4.8.   Add 2 M sodium butyrate solution to a final concentration of 2 mM. Add 1:1000 (v/v) anticlumping agent and 1:1000 (v/v) antifoam to the culture.

4.9.   Move the flask to a humidified shaker incubator at 32 °C, 90 rpm, 5% CO_2, and continue to grow for 48 h.

4.10.   Measure the cell parameters, including cell density and viability, using the auto cell counter following the user's manual.

4.11.   Transfer 2.0×10^6 cells (Vol = 2.0×10^6/cell density) in a microcentrifuge tube. Pellet the cell at 2,000 × g for 1 min in a centrifuge for western blotting in section 5.

4.12.   Harvest the cells by centrifugation at 2,000 × g for 30 min and store the cell pellet at -80 °C before purification.

5.   SDS-PAGE and western blot of HEK293 cell lysate to estimate HTT expression level

261 5.1.   Take an aliquot of 2.0×10^6 cells previously frozen (step 4.11) from the large-scale
262 transfection of HEK293 cell culture. Add 250 µL of Tris-buffered saline (TBS) supplemented with
263 50 µg/mL digitonin, 5 mM EDTA, and 1x protease inhibitor cocktail, and suspend the cell pellet
264 by aspirating several times using a pipette.
265
266 5.2.   Rotate the tubes gently for 30 min at 4 °C using a minirotator to lyse the cells. Pellet the
267 insoluble material by centrifuging at 17,000 × g for 5 min.
268
269 5.3.   Add 1/3rd the volume of 4x reducing lithium dodecylsulfate (LDS) loading buffer to the
270 supernatant and heat at 70 °C for 10 min.
271
272 5.4.   Load 5–20 µL of cell lysate onto a precast 3–8% Tris-acetate PAGE gel. Using the gel-
273 compatible 1x Tris-acetate SDS running buffer, run the gel in a constant voltage mode at 150 V
274 for 60 min.
275
276 NOTE: Tris-acetate SDS-PAGE was used for FL HTT analysis because it generates higher resolution
277 than other types of SDS-PAGE for the proteins with molecular weight above 300 kDa. Proteins
278 used in this study are also available directly from the HD Community Repository at the Coriell
279 Institute (www.coriell.org/1/CHDI); see the **Table of Materials**.
280
281 5.5.   To perform western blotting, assemble a transfer sandwich using a transfer buffer-
282 equilibrated thick transfer paper, a methanol-activated polyvinylidene fluoride (PVDF)
283 membrane, and an SDS-PAGE gel. Transfer the proteins to the PVDF membrane using a semi-dry
284 western blotter according to the manufacturer's user manual.
285
286 NOTE: Typically, 20–30 min at 135 mA is sufficient for a 10 cm x 10 cm membrane.
287
288 5.6.   Disassemble the transfer sandwich and block the membrane in TBST (20 mM Tris pH 7.4,
289 150 mM NaCl, and 0.1% v/v Tween-20) supplemented with 5% w/v non-fat milk.
290
291 5.7.   Incubate the membrane on a rocker for 1 h at room temperature with 15 mL of primary
292 antibody (1:2,500 dilution for anti-FLAG antibody monoclonal antibody and 1:2,000 for all other
293 primary antibodies).
294
295 NOTE: Primary antibodies used in this study are anti-FLAG M2, MAB5492, MAB5490, MAB2166,
296 MAB3E10, MAB4E10, MAB2168, MAB8A4 (see the **Table of Materials**).
297
298 5.8.   Wash the membrane 3 x 5 min using 30–50 mL of TBST.
299
300 5.9.   Incubate the membrane on a rocker with a fluorescent dye-conjugated goat anti-mouse
301 IgG secondary antibody at 1:15,000, room temperature, in 15 mL of TBST containing 5% w/v dry
302 milk.
303

5.10. Visualize the western blot bands on a fluorescent imager using the wavelength specific to the secondary antibody. Quantitate the band signal using the software accompanying the imager per the user's manual.

NOTE: Quantitative western blotting can be performed using purified HTT as the standard. A linear standard range of HTT is instrument-specific and was established in this laboratory from 25 ng to 250 ng of HTT per lane using an anti-FLAG antibody. The western blot of HTT should be free of degradation; a total HTT expression level of 2–4 pg/cell is typically observed. Refer to a previously published protocol[32] for details of how to perform a quantitative western blot.

6.   Fast protein liquid chromatography (FPLC) purification of HTT using anti-FLAG column and SEC

6.1.   Anti-FLAG purification

6.1.1.   Estimate the quantity of FLAG resin needed for purification (typically, 12 mL of anti-FLAG M2 affinity resin for purification of 2–4 L of transfected cell culture). Pack 12–25 mL of anti-FLAG resin onto an empty column (see the **Table of Materials**) using FPLC at a flow rate of 4 mL/min using Buffer A (**Table 1**). Adjust the height of the plunger, so there is no gap between the end of the plunger and the bed of resin.

6.1.2.   Using a ratio of 10 mL of Lysis Buffer per 1 g of cell pellet, thaw and suspend the cell pellet in cold Lysis Buffer (**Table 1**).

6.1.3.   Pass the cell suspension once through a high-shear homogenizer at 10,000 psi. Clarify the lysate by centrifugation at 20,000 × g for 1 h in a centrifuge equipped with a compatible fixed-angle rotor.

6.1.4.   Program the FPLC (see the **Table of Materials** for the software used in the study) and run the following sequences.

6.1.4.1.   Load the clarified lysate via the sample pump.

6.1.4.2.   Wash with 4 column volumes (CVs) of Buffer A (**Table 1**).

6.1.4.3.   Wash with 4 CVs of Buffer B (**Table 1**).

6.1.4.4.   Wash with 8 CVs of Buffer C (**Table 1**).

6.1.4.5.   Wash with 3 CVs of Buffer D (**Table 1**).

6.1.4.6.   Wash with 3 CVs of Elution Buffer (**Table 1**).

347 6.1.5. Analyze 10 µL of the peak fractions using SDS-PAGE. Collect and combine the peak
348 fractions with the desired purity. Save ~50 µL of the combined eluates for SDS-PAGE analysis.

350 NOTE: Normally, a single peak will appear, and all fractions eluted in the peak contain ~90% pure
351 HTT.

353 6.1.6. Regenerate an anti-FLAG column using 5 CVs of Regeneration Buffer (**Table 1**) and re-
354 equilibrate the column using 5 CVs of Buffer A.

356 NOTE: Anti-FLAG resin can be reused up to five times or until the relative yield/liter drops to 50%
357 of the first purification.

359 6.2. Size exclusion column (SEC) purification using an SEC column

361 6.2.1. Preequilibrate an SEC column that allows separation of proteins with molecular weight
362 (MW) > 500 kDa (see the **Table of Materials** for the column used) using 2 × CV of SEC Buffer
363 (**Table 1**).

365 6.2.2. Directly load the anti-FLAG eluate (from step 6.1.5) via a 50 mL superloop. Run 1.2 × CV
366 of SEC buffer per injection. Run the SEC separation overnight at 4 °C.

368 NOTE: A maximum of 5 mL or 15 mL of protein sample can be loaded on the SEC columns selected
369 in this study. Program the FPLC so that multiple injections can be performed automatically.
370 Sample method scripts are also included as **Supplemental File 1** and **Supplemental File 2**.

372 6.2.3. Compare the elution profile with the standard HTT elution profile to distinguish the
373 monomer, dimer, and higher-ordered oligomeric peaks. Pool the monomeric HTT fractions based
374 on the elution profile of the SEC column. If desired, pool the higher-ordered oligomeric and
375 dimeric HTT fractions separately.

377 6.2.4. Concentrate the pooled HTT protein using a 100 kDa centrifugal concentrator at 4 °C.
378 Calculate the protein concentrations by dividing their OD_{280} values by the respective extinction
379 coefficients (the theoretical extinction coefficients of Q23-HTT, Q48-HTT, Q73-HTT, and ΔExon1-
380 HTT are 0.776, 0.769, 0.762, and 0.798 $(mg/mL)^{-1}cm^{-1}$, respectively, for the calculation). Maintain
381 the HTT concentration ≤ 1.0 mg/mL.

383 NOTE: It is essential to monitor the concentrating process as overconcentration will result in
384 aggregation.

386 6.2.5. Aliquot the purified HTT protein in cryo-safe microcentrifuge tubes in a volume < 100 µL.
387 Flash-freeze the aliquots using liquid nitrogen and store them at -80 °C.

389 **7. Analytical HPLC SEC-MALS-dRI to analyze HTT polydispersity**

391 7.1. Perform all analytical SEC-MALS at 4 °C on a high-performance liquid chromatography
392 (HPLC) system coupled with a UV detector, a multiangle light scattering detector, and a
393 differential refractive index (dRI) detector.
394
395 7.2. Before connecting the UHPLC column to the system, purge the pump and the detectors
396 with filtered (0.1 µm) HPLC-grade water.
397
398 7.3. Connect the UHPLC column (see the **Table of Materials** for the column used) to the
399 system. Equilibrate the column with filtered (0.1 µm) water and then SEC-MALS buffer (**Table 1**)
400 until all the detector signals reach baseline.
401
402 7.4. Inject 2 µL of 6 mg/mL bovine serum albumin (BSA) at a flow rate of 0.3 mL/min for 15
403 min per injection and inspect the data quality. Perform normalization, peak alignment, and band
404 broadening correction based on the BSA profile, and create a template for the following HTT
405 sample runs.
406
407 7.5. Quickly thaw a vial of the FL Q23-HTT sample in a room-temperature water bath using a
408 float. Filter the HTT through a 0.1 µm spin filter. Inject 2–4 µL of the HTT sample and run for 15
409 min at 4 °C at a flow rate of 0.3 mL/min.
410
411 7.6. Analyze the chromatographic and light-scattering data using accompanying software (see
412 the **Table of Materials**). Use the dRI detector as a concentration detector and use 0.185 as the
413 refractive index increment (dn/dc) for HTT. Generate a Zimm plot to determine the weight-
414 averaged molecular mass for each peak[33,34].
415
416 NOTE: Refractive index increment of HTT is calculated as 0.185 using program SEDFIT software[35]
417 and primary amino acid sequence of HTT as input.
418
419 NOTE: HTT monomer MW is determined by SEC-MALS at ~370 kDa ± 30 kDa. Purified HTT typically
420 has monomer content between 60 and 75% (in this laboratory). Low monomer content may
421 indicate that more care needs to be taken in handling to prevent aggregation.
422
423 **8. Blue Native PAGE to analyze HTT polydispersity**
424
425 8.1. Prepare 1 L of Anode Buffer by mixing 50 mL of 20x Blue Native PAGE running buffer (see
426 the **Table of Materials**) with 950 mL of H_2O. Prepare 2 L of dark blue cathode buffer by mixing
427 100 mL of 20x Blue Native PAGE running buffer and 100 mL of Blue Native PAGE cathode additive
428 (20x) with 1,800 mL of H_2O. Chill the buffers to 4 °C before use.
429
430 8.2. Quickly thaw a vial of FL Q23-HTT sample in a room-temperature water bath using a float.
431 Keep the thawed protein on ice before use.
432
433 8.3. Mix 5 µg of FL Q23-HTT (~1 mg/mL), 1 µL of 0.5% G250 additive, 2.5 µL of 4x Blue Native
434 PAGE sample buffer, and water to bring the final volume to 10 µL.

8.4.	Load the mixed FL Q23-HTT sample on a 3–12% precast Bis-Tris gel. Load 7.5 µL of the unstained protein standard in the same gel as the standard.

8.5.	Fill the front of the tank with Dark Blue Cathode buffer and the back of the tank with Anode buffer.

NOTE: Fill the buffers after the sample is loaded to allow easy visualization when loading the samples.

8.6.	Run the gel at 150 V for 120 min in a cold room.

8.7.	Destain the gel with Destaining Solution (**Table 1**) until bands are observed; transfer the gel to water. Visualize and document the gel on an imaging station.

NOTE: Blue Native PAGE was originally designed to analyze membrane proteins. It was adapted in this laboratory as an alternative method to estimate HTT's monomeric content. It binds to the hydrophobic regions of HTT and prevents it from forming aggregates under buffer conditions lacking detergent. Traditional Native PAGE without using Coomassie blue G250 causes HTT to form soluble oligomers and aggregates, likely due to the many hydrophobic pockets existing in HTT.

**9.	SDS PAGE followed by Coomassie or silver staining to analyze HTT purity**

9.1.	Add 4x LDS sample buffer and 10x reducing reagent to purified FL Q23-HTT to make the final concentration of loading buffer and reducing reagent to be 1x.

9.2.	Heat the sample on a dry heating block at 70 °C for 10 min.

9.3.	Load a maximum of 1 µg of protein per well onto a 3–8% Tris acetate gel and run at 150 V for 1 h using Tris-acetate SDS running buffer.

NOTE: Proteins used in this study are also available directly from the HD Community Repository at the Coriell Institute (www.coriell.org/1/CHDI); see the **Table of Materials**.

9.4.	Coomassie stain

9.4.1.	Wash the gel with H_2O for 5 min.

9.4.2.	Stain the gel in the Coomassie staining solution (**Table 1**) by rocking the gel in 30 mL of staining solution for 15 min.

9.4.3.	De-stain by rocking the gel in 50 mL of H_2O for 5 min. Repeat twice. Visualize and document the Coomassie-stained gel on an imaging station.

479
480   9.5.   Silver stain using a commercial silver stain kit.

481

482   9.5.1.   After SDS-PAGE, fix the gel using Fixing Solution (**Table 1**) for 1 h to overnight at room
483   temperature.

484

485   9.5.2.   Perform the stain, wash, and develop according to the kit's instructions.

486

487   9.5.3.   Stop the developing step immediately once the bands reach the desired intensity.

488

489   9.5.4.   Document the gel in a gel documentation system equipped with a visible light source.

490

491   NOTE: HTT purified at >95% can be detected by Coomassie and silver staining with this protocol.
492   Refer to a previously published protocol[32] for details of how to perform quantitative protein
493   analysis.

494

495   **REPRESENTATIVE RESULTS:**
496   A transient expression vector (pcDNA3.1-Q23-*HTT*-TEV-FLAG, **Figure 1A**) is engineered for rapid
497   production in mammalian cells of FL Q23-HTT (aa 1–3,144, based on Q23 numbering). This
498   construct has the features designed to rapidly generate various HTT mutation constructs by
499   cassette cloning, facilitate purification of HTT protein to high quality and homogeneity with
500   minimal chromatographic steps, and have the option to produce untagged FL HTT. The list of the
501   features includes 1. HindIII restriction digestion sites, surrounding the CAG repeat in HTT exon 1,
502   can be used to generate FL HTT mutants with a polyQ stretch of various lengths by restriction
503   enzyme digestion and ligation; 2. the C-terminal end of FL HTT is tagged with a FLAG epitope with
504   a TEV protease recognition site for one-step affinity purification of FL HTT with high purity and
505   optional generation of tag-free FL HTT protein using TEV protease cleavage; 3. Codon-optimized
506   FL HTT sequence for human cell codon usage for high-level expression in HEK293 cells. The pcDNA
507   3.1 (+) vector is used as the backbone of the construct to take advantage of the high
508   transcriptional activation activity of the CMV promoter in mammalian cell lines.

509

510   Using pcDNA3.1-Q23-*HTT*-TEV-FLAG as the starting template, the Q48 and Q73 FL HTT constructs
511   were produced by synthesizing DNA fragments with proper Q length spanning two HindIII
512   restriction enzyme sites and swapping the same region in the template. The ΔExon1 mutant of
513   FL HTT (aa 91–3,144) (**Figure 1B**) was produced using primers directed to deleted residues
514   spanning the exon 1 region in the template. HEK293 cells transfected with pcDNA3.1-Q23-*HTT*-
515   TEV-FLAG using PEI were grown in 5 L shaker flasks under 5% CO_2. A typical large-scale
516   purification uses a 2–10 L cell pellet containing 6.0×10^9–3.0×10^{10} cells. Before proceeding to
517   purification, the HTT expression level from each transfection was estimated by quantitative
518   western blotting using purified recombinant FLAG-tagged HTT as a standard and anti-FLAG
519   antibody as the first antibody. Pellets with an estimated HTT expression level at ≥2 pg HTT/cell
520   were used for purification.

521

522   Purification of FL HTT consists of a 2-step column process, first with anti-FLAG affinity purification
523   and then with SEC on a gel filtration column with a suitable separation range for HTT (**Figure 2A;**
524   see **Table of Materials** for examples). After both steps, HTT was obtained at >95% sample purity,
525   as determined by SDS-PAGE with Coomassie blue and >65% monomer content based on
526   analytical SEC-MALS. Because both prolonged purification time and temperature have a negative
527   impact on the final HTT monomer content, FPLC was used in both purification steps to minimize
528   handling and obtain consistent sample quality. The major contaminant during the anti-FLAG
529   purification was the chaperone Hsp70 as determined by mass spectrometry (**Figure 2B**, lane 2).
530   This is consistent with the finding that Hsp70 is co-purified with FL HTT stably expressed in human
531   cell lines[24], suggesting that Hsp70 may be a common stabilizer for FL HTT *in vivo*.

533   Hsp70 contamination can be eliminated by extensively washing with magnesium chloride and
534   ATP during the anti-FLAG affinity purification step (**Figure 2B**, lane 1). Upon removal of Hsp70, FL
535   HTT is prone to form higher-ordered oligomers[24] and has to be maintained at a concentration ≤
536   1 mg/mL. The concentration step before SEC can often result in significant aggregation. Therefore,
537   the best practice is to directly load peak fractions from anti-FLAG purification onto the size
538   exclusion column without concentrating. After SEC, the sample was concentrated to ≤1 mg/mL
539   for maximum recovery of monomeric FL HTT. The amount of HTT recovered from each
540   purification step was estimated by either Coomassie blue or quantitative western blotting using
541   purified FL HTT as a quantification standard (**Table 2**). The typical yield of purified FL HTT proteins
542   produced by the described method is approximately 1 mg/L of cell culture but may fall well below
543   that (**Table 3**) due to batch-to-batch variability, or if the anti-FLAG purification resin is reused
544   multiple times.

546   Overexpression of FL HTT can result in fragmentation of the protein[22]. FL Q23-HTT produced by
547   the method described here resolved as a single band with the correct MW of 350 kDa by SDS
548   PAGE, stained either by Coomassie G250 or by silver staining (**Figure 2C**). By western blotting, FL
549   Q23-HTT reacted with antibodies raised against epitopes at the N-terminal, C-terminal, and
550   several intermediate domains, with no additional fragment-related bands observed, indicating
551   that the protein was isolated without significant detectable truncations (**Figure 3A**). FL HTT polyQ
552   length variants Q23, Q48, and Q73 reacted as expected in western blot, showing a progressively
553   stronger signal for polyQ-directed mAb MW1 correlating with increasing Q-length: Q23-HTT <
554   Q48-HTT < Q73-HTT (**Figure 3B**). No signal was observed for ΔExon1-HTT (aa 91–3,144) when
555   probed with the antibodies MW1 and MAB549, which target the N terminal exon 1 (**Figure 3B**).

557   SEC-MALS was employed to analyze the aggregation state and molecular mass of the purified
558   HTT protein. Samples were analyzed by analytical SEC monitored by UV, MALS, and dRI detectors.
559   The absolute molar mass obtained from SEC-MALS does not depend on the shape of
560   molecules[33,34]; therefore, SEC-MALS provides an unbiased estimation of MW for monomeric and
561   oligomeric fractions when they are well separated. Among the HPLC columns tested, the SEC
562   column (see the **Table of Materials**) showed sufficient resolution between the HTT monomer and
563   dimer such that molar masses could be distinguished (**Figure 4**). The protein concentration was
564   determined by dRI detection. Refractive index increments (dn/dc) of FL HTT are 0.1853 mL/g as
565   calculated by the SEDFIT software[35]. Similar analytical SEC elution patterns were observed for

566 ΔExon1 HTT (91–3,144), FL Q23, Q48, and Q73 HTT (1–3,144), each consisting of a major
567 monomer peak with minor dimeric and oligomeric peaks (**Table 4**). The calculated MW for the
568 monomeric form is greater than the theoretical MW. This is probably caused by overlapping
569 species from higher-ordered oligomeric peaks and errors resulting from weak dRI signals as HTT
570 proteins are maintained in low concentration to avoid forming higher-ordered oligomers. By
571 integrating the UV peaks of several batches of purified FL HTT variants, no clear correlation
572 between polyQ length and aggregate profile was observed (**Table 4**).

574 In addition to analytical SEC, traditional native PAGE was performed to determine whether it can
575 be used as a complementary method to characterize the FL HTT oligomeric state. Higher-ordered
576 oligomers were resolved through 3–8% Tris-acetate gels using native buffer without detergent.
577 Purified FL HTT from SEC showed multiple bands corresponding to the oligomerization states
578 (**Figure 5A**). The lowest band was located between native marker 480 kDa and 720 kDa, similar
579 to previous results reported for FL HTT purified from insect cells[22]. However, the HTT monomer
580 was not the most abundant band when using traditional native PAGE, and the results do not
581 correlate with the aggregate profile determined by analytical SEC-MALS. Several hydrophobic
582 patches present in FL HTT[36–38], especially the hydrophobic interface between HAP40 and FL HTT[25],
583 are likely to contribute to the formation of higher-ordered oligomers during migration within the
584 gel. This is because hydrophobic regions are known to interact with each other in the absence of
585 detergent or stabilizing protein-protein interactions. Consistent with the hydrophobic properties
586 of HTT, FL HTT forms increasing amounts of higher-ordered oligomeric fractions in the absence
587 of CHAPS during the SEC purification step.

589 Blue Native PAGE, which is widely used to investigate membrane proteins and large protein
590 complexes containing hydrophobic patches[39], was compared to traditional native PAGE. Purified
591 HTT showed three major bands on Blue Native PAGE with estimated MW of 643, 927, and 1070
592 kDa (**Figure 5B**) that likely represent the monomeric, dimeric, and trimeric species of HTT,
593 respectively. The monomer band remained the most abundant band in the Blue Native PAGE,
594 corresponding well to the analytical SEC profile of the same samples. The overestimation of MW
595 of the HTT monomer by Blue Native PAGE may result from the unique hollow spherical structure
596 or hydrophobic regions of HTT that cause slower migration relative to corresponding molecular
597 weight markers[11,23,25]. Overall, FL Q23-HTT, FL Q48-HTT, FL Q73-HTT, and ΔExon1-HTT have
598 similar Blue Native PAGE profiles with only slight differences in the protein band migration due
599 to their molecular weight differences.

601 As an additional check of the quality of the purified proteins, the C-terminal FLAG tag can be
602 removed from FL HTT by treatment with TEV protease. After proteolytic cleavage, samples were
603 analyzed by western blot using four antibodies to confirm FLAG tag removal and detect HTT
604 degradation. Immunoreactivity to anti-FLAG M2 and three huntingtin-specific antibodies with
605 epitopes to the N-terminus, intermediate domains, and C-terminus of HTT showed successful
606 FLAG tag removal and no HTT-specific degradation products (**Supplemental Figure S1**).

608 **FIGURE AND TABLE LEGENDS:**

Figure 1: Construct for full-length HTT expression. (**A**) Full-length Q23 *HTT* was codon-optimized and cloned into pcDNA3.1 (+) plasmid. The 3' end of HTT was tagged with Flag epitope and TEV protease cleavage site to produce tag-free HTT protein. The polyglutamine stretch and proline-rich domain were engineered with flanked HindIII restriction endonuclease sites to insert additional CAG repeats using cassette cloning, *i.e.,* Q48 and Q73, to produce HTT variants with different polyQ lengths. (**B**) ΔExon1 construct was made PCR mutagenesis using pcDNA3.1-Q23-HTT as the template. Residues 91–3,144 of HTT remained in the ΔExon1 construct for expression. Abbreviations: HTT = huntingtin; CMV = cytomegalovirus; Q23 = polyglutamine stretch; PRD = proline-rich domain; TEV = tobacco etch virus cleavage site.

Figure 2: Large-scale purification of HTT. (**A**) SEC profile of anti-Flag-purified full-length Q23-HTT on an FPLC column. High-ordered oligomers, dimer, and monomer peaks of Q23-HTT are labeled. Fractions containing monomer were collected as the final HTT sample. (**B**) SDS-PAGE of purified Q23-HTT with ATP/magnesium washing step (lane 1) or without ATP/magnesium washing results in Hsp70 co-elution (lane 2). (**C**) Final purified full-length HTT variants on SDS-PAGE stained with Coomassie blue G-250 or silver stain. Abbreviations: FL = full length; HTT = huntingtin; SEC = size exclusion chromatography; FPLC = fast protein liquid chromatography; O = oligomer; D = dimer; M = monomer; SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis; Hsp70 = heat shock protein 70.

Figure 3: Western blot analysis of purified HTT Variants. (**A**) Purified FL Q23-HTT was run on SDS-PAGE and transferred to PVDF membrane. The primary antibodies and interacting epitopes are Lane 1, α-FLAG M2, FLAG tag; Lane 2, MAB5492, HTT aa. 1–82; Lane 3, MAB5490, HTT aa 115–129; Lane 4, MAB2166, HTT aa 181–810; Lane 5, MAB3E10, HTT aa 1,171–1,177; Lane 6, MAB4E10, HTT aa 1,844–2,131; Lane 7, MAB2168, HTT aa 2,146–2,541; Lane 8, MAB8A4, HTT aa 2,703–2,911. (**B**) 1 μg of purified FL HTT variants were run on SDS-PAGE and transferred to PVDF (left), and a duplicate SDS gel was run and stained with Coomassie Blue (right). The primary antibodies and interacting epitopes are Row 1, MW1, expanded PolyQ repeats; Row 2, MAB5492, HTT aa 1–82; Row 3, MAB2166, HTT aa 181–810. Abbreviations: FLL Q23-HTT = full-length huntingtin protein containing 23 glutamine residues; SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis; WB = western blot; M = marker; PVDF = polyvinylidene fluoride.

Figure 4: SEC-MALS analysis of full-length HTT. Purified full-length Q23-HTT was eluted on a UPLC column. Peak positions of predicted monomer, dimer, and oligomer are indicated. Molecular weights were calculated for monomer, dimer, and trimer peaks and listed in **Table 5**. Similar elution profiles are observed for Q48, Q73, and ΔExon1 HTT, with variable monomer, dimer, and oligomer contents in each purification. Abbreviations: SEC-MALS = Size exclusion chromatography with multi-angle light scattering; UV = Ultra Violet; LS = Light Scattering; MW = Molecular Weight; Q23-HTT = huntingtin protein containing 23 glutamine residues; M = monomer; D = dimer; O = oligomer.

Figure 5: Characterization of purified HTT using clear Native PAGE or Blue Native PAGE gel. Native Marker and apparent monomeric Q23-HTT from SEC were resolved on 3–8% Tris-acetate

15

gels in a non-denaturing PAGE system (**A**) and a Blue Native PAGE system (**B**). Abbreviations: FL = full-length; Q23-HTT = huntingtin protein containing 23 glutamine residues; PAGE = polyacrylamide gel electrophoresis; M = marker.

Table 1: Composition of buffers and solutions

Table 2: HTT yield from a 2 L HEK293 pellet transfected with pcDNA3.1-Q23-*HTT*-TEV-Flag. Abbreviations: FL Q23-HTT = full-length huntingtin protein containing 23 glutamine residues; TEV = tobacco etch virus cleavage site; SEC = size exclusion chromatography.

Table 3: Summary of the protein yield of four FL HTT variant purifications and their final purity. Abbreviation: FLL HTT = full-length huntingtin protein.

Table 4: Summary of the representative aggregate, dimer, and monomer content of FL HTT variants from the purification. Abbreviations: FL HTT = full-length huntingtin protein; A = aggregate; D = dimer; M = monomer; SEC = size exclusion chromatography.

Supplemental Figure S1: Western blot analysis following TEV protease digestion. Purified FL Q23-HTT and FL Q48-HTT were run on SDS-PAGE, transferred to PVDF membranes, and analyzed by western blotting following TEV digest. Primary antibodies used were anti-Flag M2 (Flag tag), MAB5492 (HTT aa 1–82), MAB3E10 (HTT aa 997–1,276), and MAB2168 (HTT aa 2,146–2,541). Lane 1, Protein Standard; Lane 2, Q23-HTT-TEV-Flag; Lane 3, Q48-HTT-TEV-Flag; Lane 4, Q23-HTT-TEV-Flag treated with TEV protease at 1:5, overnight at 4 °C; Lane 5, Q48-HTT-TEV-Flag treated with TEV protease at 1:5, overnight at 4 °C. Abbreviations: FL HTT = full-length huntingtin protein; SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis; TEV = tobacco etch virus; PVDF = polyvinylidene fluoride.

Supplemental Figure S2: SEC-MALS analysis of FL HTT variants subjected to freeze-thaw cycles. Purified Q23-HTT (**A**) and Q48-HTT (**B**) were frozen at -80 °C and thawed at room temperature for up to 6 times. Q23-HTT and Q48-HTT after the first freeze-thaw and the sixth freeze-thaw cycles were then analyzed by SEC-MALS. A slight decrease in monomer fraction and increase in dimer and high-order oligomer fractions were observed by light scattering after repeated freeze-thaw cycles. Peak positions of predicted monomer, dimer, and high-order oligomer are indicated. Abbreviations: FL HTT = full-length huntingtin protein; O = oligomer; D = dimer; M = monomer; SEC-MALS = Size exclusion chromatography with multi-angle light scattering.

Supplemental Figure S3: SDS PAGE of FL HTT variants subjected to freeze-thaw cycles. Purified Q23-HTT (**lanes 2–7**) and Q48-HTT (**lanes 9–14**) were frozen at -80 °C and thawed at room temperature for up to 6 times. Aliquots of Q23-HTT and Q48-HTT were stored after each freeze-thaw cycle and then analyzed by SDS PAGE. No increase in aggregate or degradation products was observed; the samples were considered stable and >95% pure by band densitometry. Abbreviations: FL HTT = full-length huntingtin protein; SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis.

697 **Supplemental File 1: FPLC 15 mL anti-FLAG HTT script.** Abbreviations = FPLC = fast protein liquid
698 chromatography; HTT = huntingtin protein.
699
700 **Supplemental File 2: FPLC SEC_MALS HTT script.** Abbreviations: SEC-MALS = Size exclusion
701 chromatography with multi-angle light scattering; FPLC = fast protein liquid chromatography; HTT
702 = huntingtin protein.
703
704 **DISCUSSION:**
705 We describe here a transient transfection, expression, and purification method to generate
706 multiple FL HTT protein constructs with suitable purity and homogeneity for use as standards for
707 immunoassay and MS assay development, controls for western blot analysis, and for structure–
708 function studies. This transient expression method is scalable and versatile and enables the user
709 to generate low-milligram quantities of FL HTT variants more efficiently than using stable cell
710 lines or virus-based methods described previously[21–24]. Routinely, 2–5 mg of highly purified FL
711 HTT can be generated from a 2 L scale protein production run in less than a week using the
712 transient expression method once the plasmid is constructed, with a typical yield of 1–2.5 mg of
713 FL HTT per liter of cell culture.
714
715 The transient expression method described here overcomes many obstacles in stable cell line
716 expression, such as the long time needed to establish cell lines and difficulties in storage and
717 maintaining stable cell lines. PEI is also relatively inexpensive compared to other transfection
718 reagents in the market, making large-scale transfection economically viable. There are also
719 limitations in the protocol: transfection efficiency largely depends on the quality of the plasmids,
720 optimal cell growth, and how well PEI is stored and prepared. Operators need to take special care
721 and perform quality controls in those critical steps to avoid a drastic drop in protein yields. Anti-
722 FLAG resin used in the protocol is also relatively expensive and shows reduced capture of FL HTT
723 after several purifications and regenerations. Some researchers may find it more practical to
724 switch to a different tag to allow more robust regeneration of the affinity resin.
725
726 Various cell lines and expression conditions were tested to optimize FL HTT expression levels.
727 HEK293 cells were chosen for the expression of FL HTT because of the high expression of protein
728 and the ease of handling in a suspension culture format, making the method suitable for large-
729 scale expression in either shakers or bioreactors. A higher FL HTT protein expression level can be
730 achieved at lower culturing temperatures such as 32 °C rather than using the customary
731 temperature of 37 °C. It is possible that the lower temperature may slow protein synthesis and
732 promote correct folding of FL HTT[40]. However, this phenomenon is not specific to FL HTT or the
733 cell lines tested. The reduced posttransfection temperature has been widely used in
734 pharmaceutical protein expression in CHO cells. Although the mechanism is not fully understood,
735 it is thought that low temperatures arrest the cell cycle in the G1 phase and divert cellular energy
736 to protein production[41].
737
738 Full-length HTT purified from mammalian cells co-elutes with the chaperone Hsp70[24], and Mg–
739 ATP washing steps can remove the Hsp70 protein. Interestingly, co-eluted Hsp70 is not observed
740 in FL HTT purified from an insect cell expression system[21–23]. This may reflect a difference in the

741   PTMs of FL HTT or heat shock protein responses to the overexpression of FL HTT in mammalian
742   and insect cells. Once the recombinant protein has been stripped of Hsp70, non-ionic detergents
743   such as CHAPS or DDM are required to stabilize the monomeric form of FL HTT.
744
745   The oligomerization states of FL HTT variants were analyzed using Blue Native PAGE and SEC-
746   MALS. A small fraction of dimeric and higher-order oligomeric HTT was present when analyzed
747   by either Blue Native PAGE or SEC-MALS. Of note, higher-ordered oligomers formed by FL HTT
748   do not appear to correlate with polyQ length, and even the Exon1 deletion mutant displays a
749   similar oligomer-dimer-monomer ratio. The actual variations in oligomer content among these
750   constructs are likely due to minor differences in the production and handling of each batch. In
751   contrast to aggregates and fibrils formed by HTT Exon1[42,43], the higher-ordered oligomers of FL
752   HTT remained soluble and could be analyzed by SEC and Native PAGE.
753
754   Purified monomeric FL HTT is only relatively stable. Prolonged storage at 4 °C, short incubations
755   at room temperature, or concentrations > 1 mg/mL will all convert monomeric FL HTT to dimeric
756   and higher-ordered oligomeric forms even though there is no visible precipitation observed
757   under those conditions. Purified monomeric FL HTT maintained at ≤1 mg/mL remained relatively
758   stable at -80 °C in storage buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 5% v/v glycerol, 0.5% w/v
759   CHAPS, and 5 mM DTT) as previously described[24]. Up to 6 freeze-thaw cycles of FL HTT prepared
760   and stored in this manner did not cause visible precipitation of the protein, although a slight shift
761   to a higher oligomeric state was observed by SEC-MALS (**Supplemental Figure S2**). Samples were
762   also analyzed by SDS PAGE following repeated freeze-thaw cycles. No visible precipitates were
763   observed; no aggregates or additional degradation products were seen by SDS-PAGE
764   (**Supplemental Figure S3**). The long-term stability of purified FL HTT is still under investigation. In
765   the absence of conclusive long-term data, we recommend storing purified FL HTT at -80 °C for no
766   longer than 6 months.
767
768   High-quality, recombinant FL HTT protein variants and the methods to produce them are in high
769   demand by the HD research community. These proteins are in use as immunoassay and MS
770   analytical standards, in structural studies, and for the development of novel FL HTT-specific
771   assays. The large-scale transient expression methods described here have consistently produced
772   milligram quantities of FL HTT variants with >95% purity, providing essential tools for HTT studies.
773   Production of tens of milligrams of highly purified FL HTT polyQ variants and other mutants in
774   support of HD research has become routine.
775

783
784   **DISCLOSURES:**

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887
888

Figure 1

Figure 1

A

B
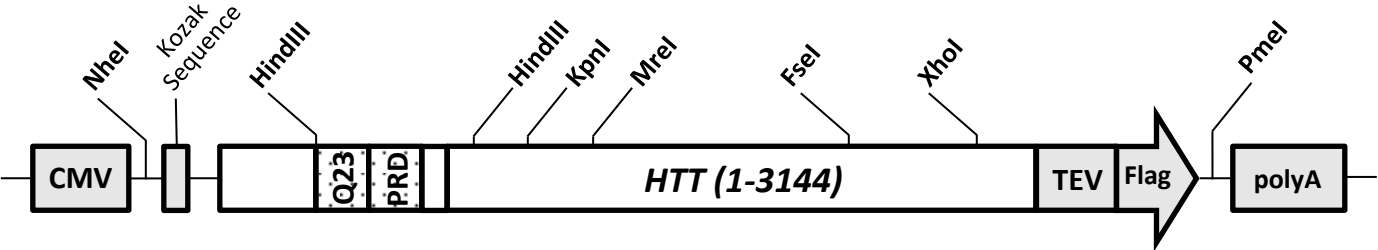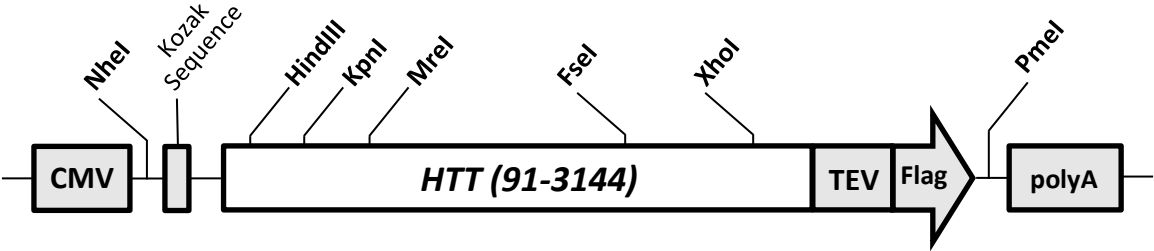
Figure 2

Figure 2

A
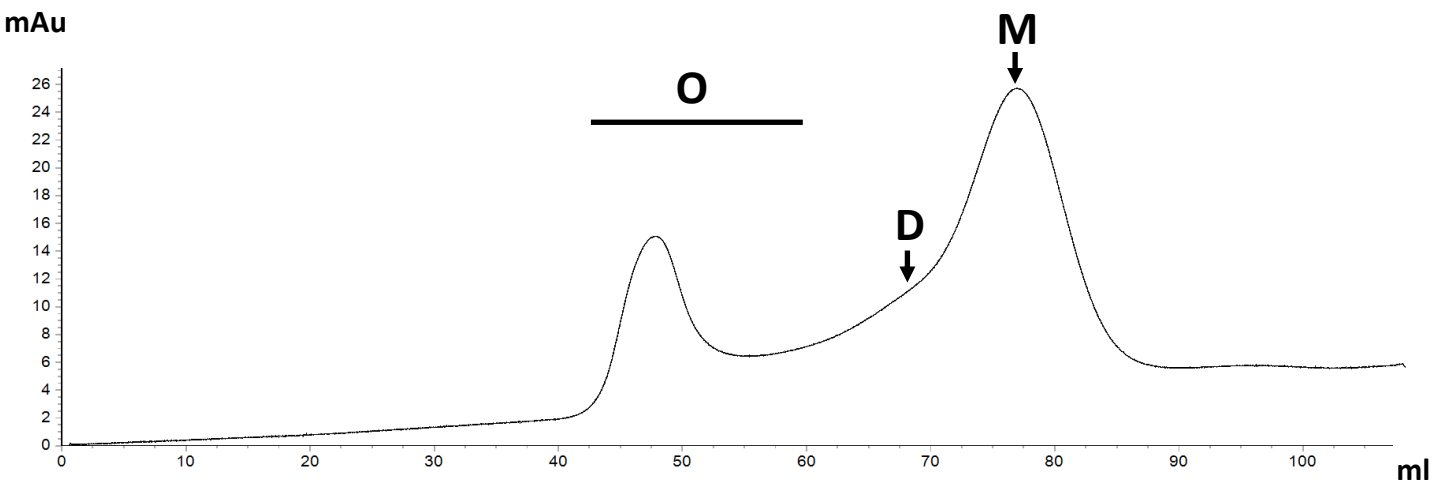
Figure 2

B

Figure 2

C

Figure 3

Click here to access/download;Figure;Figure 3.pdf ⬇

Figure 3

A

1 2 3 4 5 6 7 8

FL Q23-HTT

Figure 3

B

WB   Coomassie

Figure 4
Click here to access/download;Figure;Figure 4 (2).pdf ⬇
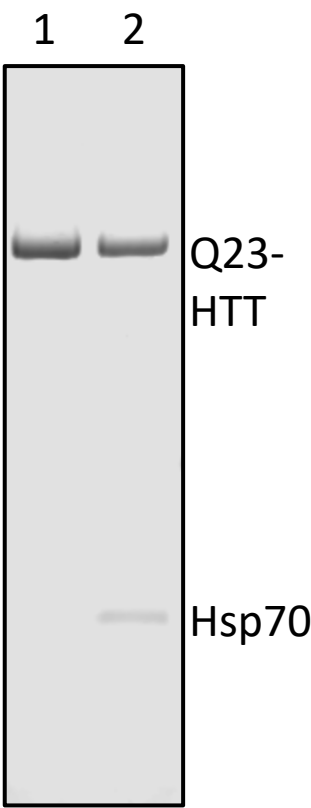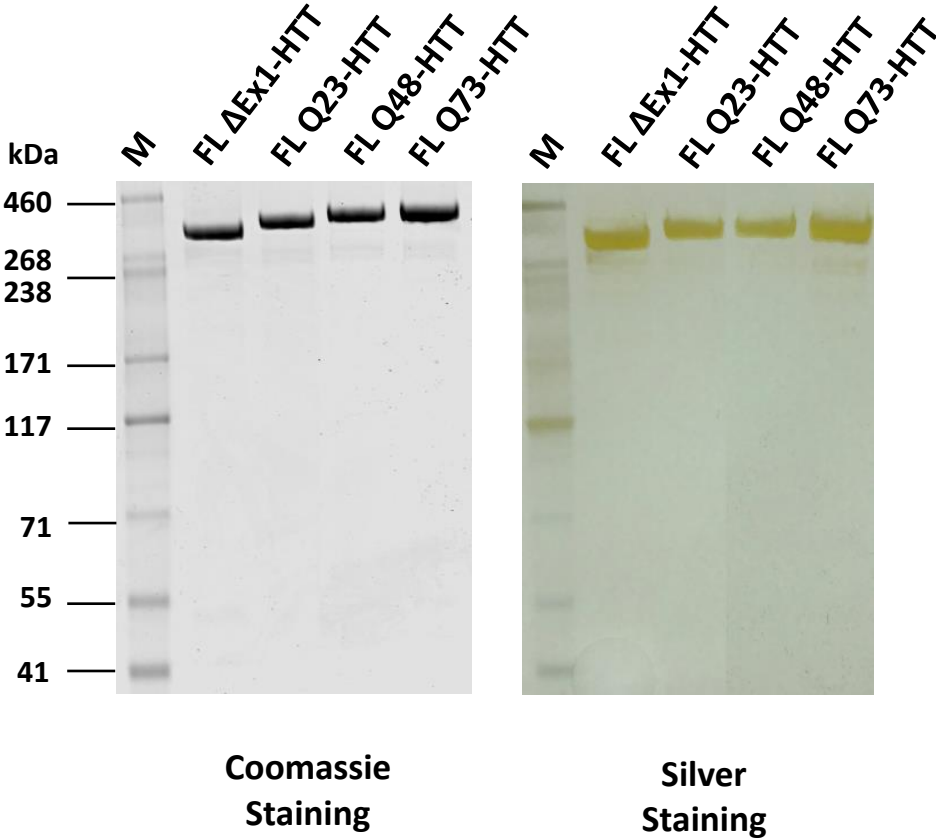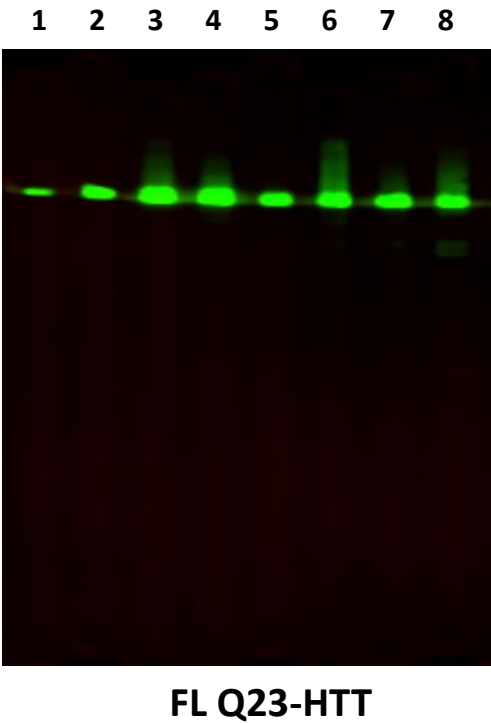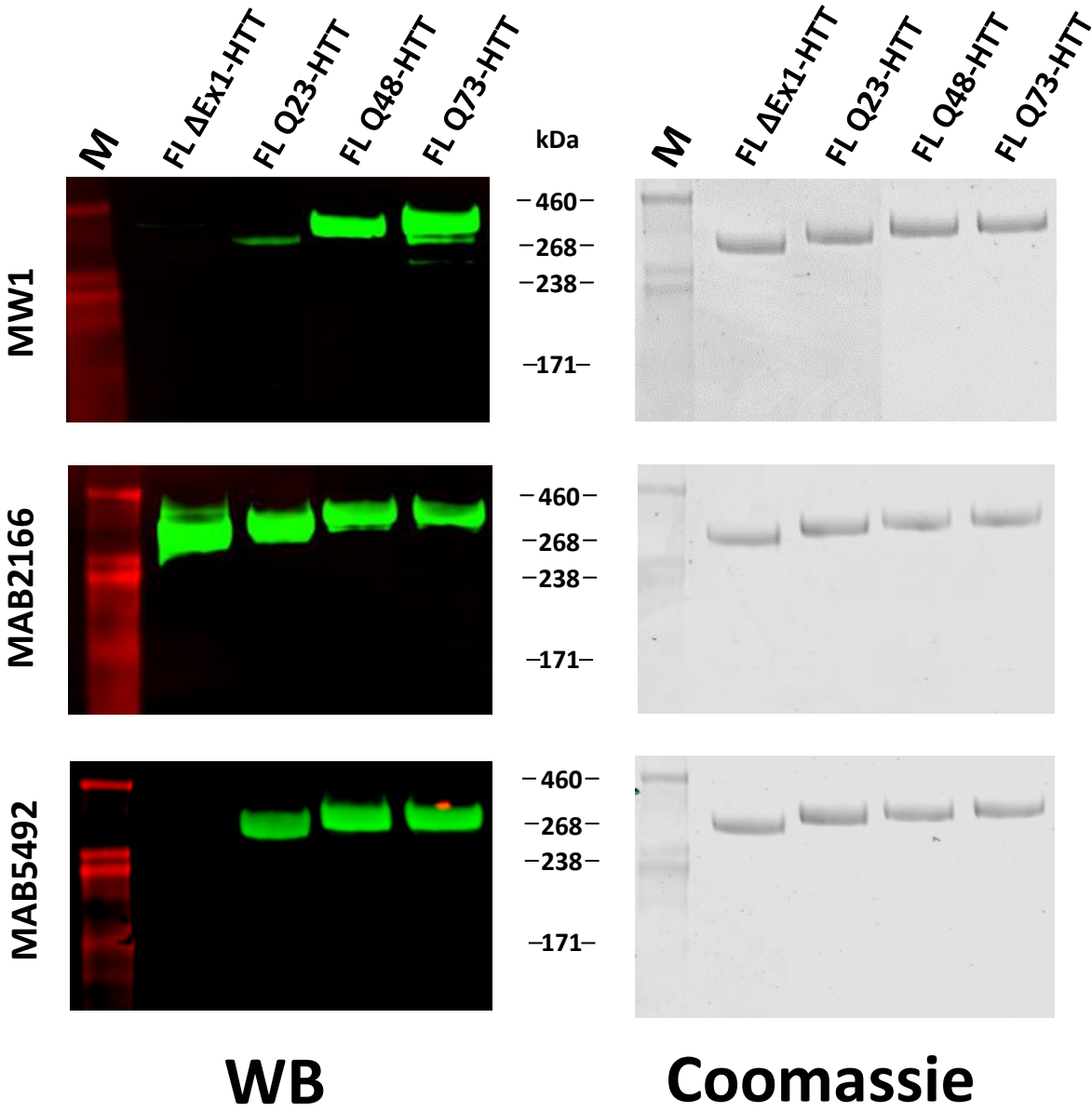
Figure 5

Figure 5

Figure 4

A

Figure 4

B

Table 1

Click here to access/download;Table;Table 1 (2).xlsx ⬇
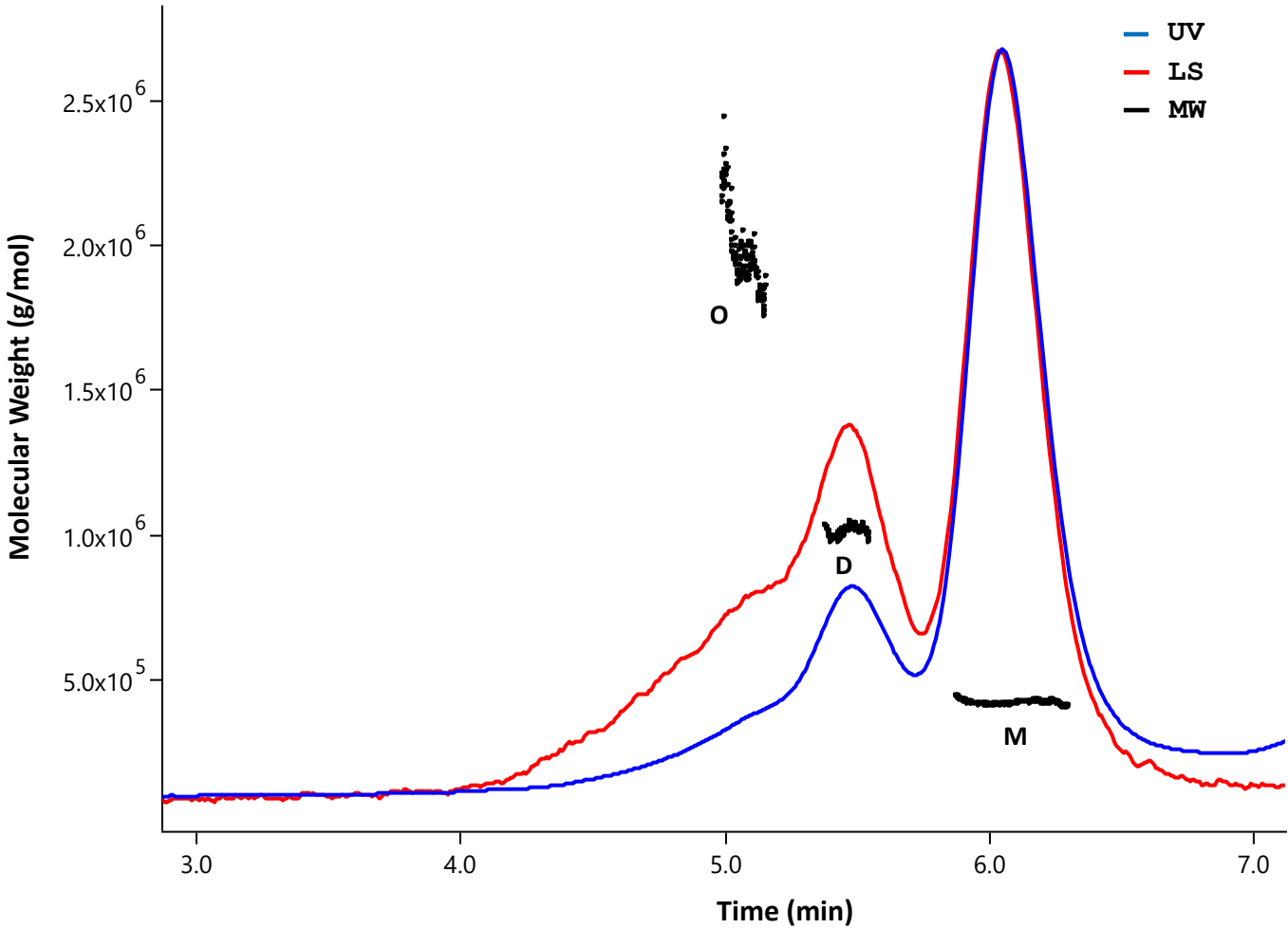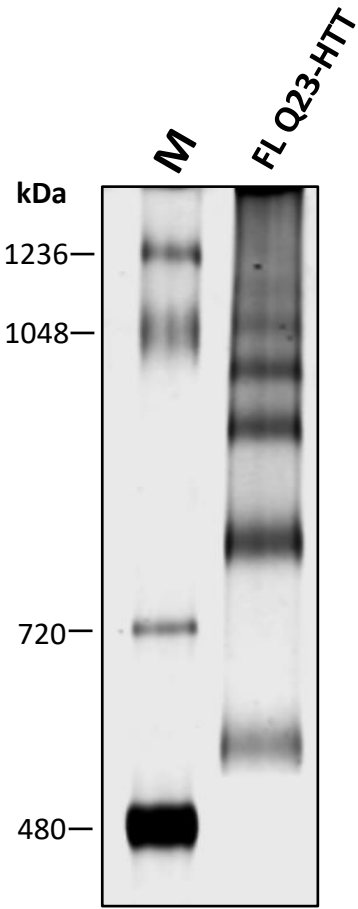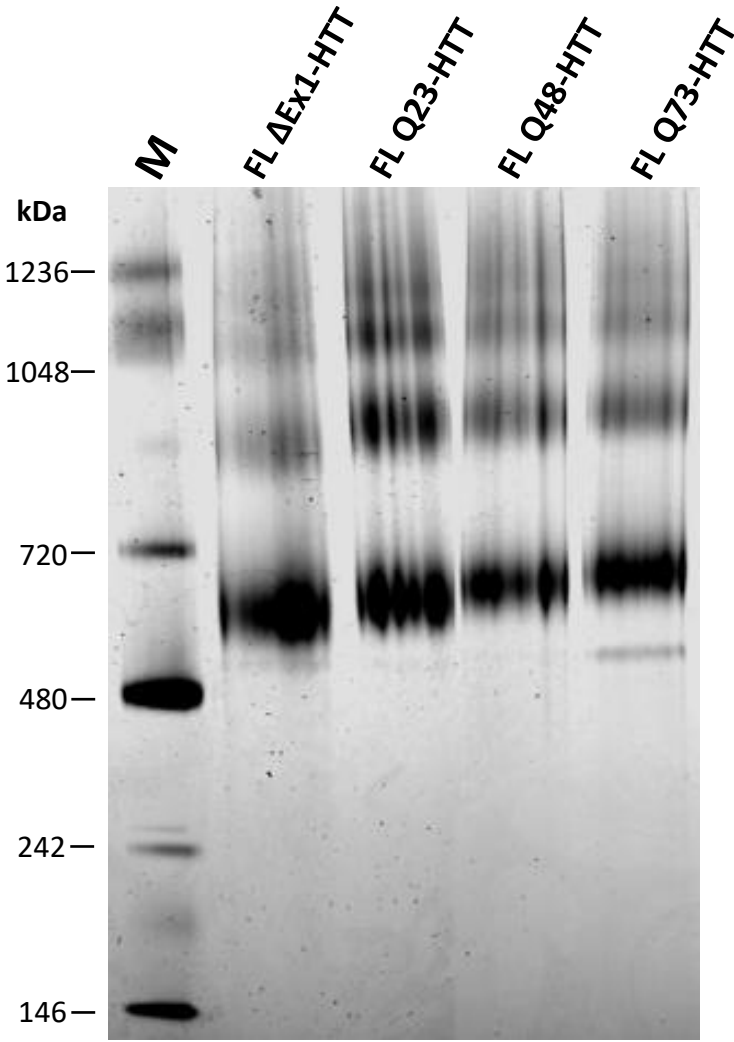
Step	Name	Composition
6.1.1	Buffer A	50 mM Tris, 500 mM NaCl, 5% v/v glycerol, 5 mM EDTA, 0.01% v/v Tween-20, pH 8.0.
6.1.2	Lysis Buffer	50 mM Tris, 500 mM NaCl , 5% v/v glycerol, 5 mM EDTA, and 1x protease inhibitor cocktail
6.1.4.2	Buffer A	50 mM Tris, 500 mM NaCl, 5% v/v glycerol, 5 mM EDTA, 0.01% v/v Tween-20, pH 8.0.
6.1.4.3	Buffer B	50 mM Tris; 500 mM KCl; 5 mM MgCl2; 5% v/v glycerol; 0.01% v/v Tween-20, pH 8.0
6.1.4.4	Buffer C	20 mM Tris; 200 mM KCl; 5 mM MgCl2; 5 mM ATP; 0.01% v/v Tween-20; 5% v/v glycerol, pH 8.0
6.1.4.5	Buffer D	50 mM Tris; 500 mM NaCl; 5% v/v glycerol; 5 mM EDTA; 0.5% w/v CHAPS, pH 8.0
6.1.4.6	Elution Buffer	50 mM Tris; 500 mM NaCl; 5% v/v glycerol; 0.5% w/v CHAPS; 0.2 mg/mL DYKDDDDK peptide, pH 8.0
6.1.6	Regeneration Buffer	0.1 M glycine HCl, pH 3.5; 0.01% v/v Tween-20
6.2.1	SEC Buffer	50 mM Tris, 500 mM NaCl, 5% v/v glycerol, 0.5% w/v CHAPS, 5 mM dithiothreitol [DTT]
7.3	SEC-MALS Buffer	50 mM HEPES, pH 7.2, 500 mM NaCl, 5% v/v glycerol, 0.5% w/v CHAPS
8.7	Destaining Solution	40% v/v methanol and 7% v/v acetic acid
9.4.2	Coomassie Staining Solution	0.01% w /v Coomassie G250, 50% v/v/ methanol, 10% v/v acetic acid
9.5.1	Fixing Solution	50% v/v methanol , 10% v/v acetic acid, 50 µL of formaldehyde/100 mL of solution

Table 2

Steps	HTT concentration (mg/ml)	Total Volume (mL)	HTT content (mg)	HTT yield per cell (pg/cell)	% Yield
Supernatant	0.1792	220	39.4	4.4	100
Anti-Flag	1.524	8.6	13.1	1.47	33.4
SEC	0.91	3.9	3.54	0.4	9.1

Table 3

HTT Sample		HTT Yield (mg/L)		Avg Purity (%)
		BCA	A_{280}	
1	FL DEx1-HTT (N=3)	0.67-1.30	0.69-1.18	99.3
2	FL Q23-HTT (N=3)	0.25-0.92	0.28-0.98	96.9
3	FL Q48-HTT (N=3)	0.28-1.15	0.38-1.16	97.4
4	FL Q73-HTT (N=3)	0.58-1.05	0.57-0.97	98.8

Table 4

HTT Sample	A	D	M
FL Q23-HTT	4.2-6.9%	18.7-29.3%	66.5-76.0%
FL Q48-HTT	4.0-9.4%	10.6-17.8%	73.6-85.4%
FL Q73-HTT	2.0-14.0%	16.9-24.6%	65.1-81.1%

Click here to access/download
Table of Materials
Table of Materials (1).xlsx

Author Rebuttal Letter: Pace et al. JoVE63190

<u>Editorial comments:</u>

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Revised according to editor's comment.

2. Line 57: Please number the references in the order in which they appear in the text. So, 41-43 should be numbered 18-20 instead and so on.

Reference number was adjusted according to editor's comment.

3. Use SI units as much as possible and abbreviate all units: L, mL, µL, cm, kg, etc. Use h, min, s, for hour, minute, second.

Revised throughout manuscript according to editor's request.

4. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example, NanoDrop, Ultra Yield, EndoFree, QIAGEN, Endosafe Nexgen-PTS, Milli-Q, Expi293, ViCell XR (Beckman Coulter), EX-CELL, Sigma Aldrich, ProBlock, NuPAGE, IRdye 800, Odyssey CLx, Sorvall Lynx 6000, A23-6x100, GenScript, Agilent 1260 Infinity II Bio-Inert, Waters, Astra 7 (Wyatt Technology), Eppendorf, NativePAGE Novex, SEDFIT, etc.

Commercial information was moved to material table according to the instructions. SEDFIT is a program made by NIH and freely available.

7. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
Step 2.2: How was the ligated product transformed? Was heat-shock transformation used? If yes, please provide all associated steps. How much LB was added? How was the colony Miniprep done? How was the glycerol stock made? Please provide all associated steps. Alternatively, add references to published material specifying how to perform the protocol action.

Details added according to the manufacturer's instructions.

Step 3.6: How was the endotoxin level measured? Please provide all the associated steps.

Added manufacturer instruction reference and also in the material table.

Step 4.2: How much concentration of penicillin/streptomycin does 0.5x penicillin/streptomycin denote? How were the cells counted? What was the dilution made in: the expression medium or any other medium? Please specify.

Step 4.3: What is the cell density used here for transfection? How was the cell density and viability assessed and calculated using the cell counter and analyzer? Please mention all the steps that are necessary to execute the action item. If this step needs to be filmed, please make sure to provide all the details such as "click this", "select that", "observe this", etc. Please provide details so a reader may replicate your analysis, including buttons clicked, inputs, screenshots, etc. Please remember that software steps without a graphical user interface (GUI) cannot be filmed.

Step 5.4: How was the Western blotting done? How was the blot quantified? Please provide all associated steps. Alternatively, add references to published material specifying how to perform the protocol action.

Step 5.5: How were the proteins transferred to the PVDF membrane? How much TBST was used for blocking? With what solution was the membrane washed? Please provide the volume of all the antibodies added.

Step 5.6: What steps were followed to visualize the blot, and how were the expression levels quantitated (normalization, the formula used, etc.)? Please mention all the steps that are necessary to execute the action item. If this step needs to be filmed, please make sure to provide all the details such as "click this", "select that", "observe this", etc. Please provide details so a reader may replicate your analysis, including buttons clicked, inputs, screenshots, etc. Please remember that software steps without a graphical user interface (GUI) cannot be filmed.

Step 6.1.5: How was the SDS-PAGE run? What % SDS-PAGE was made, including the voltage, run time, etc. Please provide all associated steps. Alternatively, add references to published material specifying how to perform the protocol action.

Step 6.2.2: How was the multiple injection program made? Please include all the button clicks, command lines, etc. in the software. If using long scripts and long command, please include as a supplementary file. We need actions to show how the software is used.

Because Unicorn is commercial software, a general instruction was given for FPLC operation in the protocol section without mention its specific GUI. The detailed method script is added to the supplemental file.

Step 6.2.3: How do you distinguish between monomeric, dimeric, and higher-order oligomeric elutes?

Descriptions added "Compare the elution profile with standard HTT elution profile to distinguish monomer, dimer and higher-ordered oligomeric peaks." Monomer, dimer and oligomer peaks will have the similar elution volume in the SEC profile.

Step 6.2.4: What is the formula/equation used for the calculation of concentration. What was the HTT protein dissolved in after concentrating on obtaining the mentioned concentration?

Formula added. The HTT remained in the same buffer as described in the SEC section 50 mM Tris, 500 mM NaCl, 5% v/v glycerol, 0.5% w/v CHAPS, 5 mM DTT during concentrating process.

Step 8: How was the staining with Coomassie done? Please provide the volume, concentration of the stain, and staining time. What was used as a de-stain? How was the analysis done? Please provide all associated steps. Alternatively, add references to published material specifying how to perform the protocol action.

Details added. Removed gel analysis as it is not essential to the current protocol.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Revised according to the comment.

9. Please include a single line space between each step, substep, and note in the protocol section.

Revised according to the comment.

10. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Revised according to the comment.

11. Please submit each figure individually in a high-resolution format to your Editorial manager account.
12. All tables should be uploaded separately to your Editorial Manager account in a .xls or .xlsx file.
13. Please include all the Figures and table Legends for main figures and supplementary figures together at the end of the Representative Results in the manuscript text.
14. Figure 2B: The panel is slightly cut, and the labeling is not completely visible. Please ensure that everything is visible in the figures.

15. Figure 4: What does UV, LS, MW stand for? Please define all abbreviations in figure legends.
16. Figure Supplementary 2SA: There are overlapping markings on the curve. Please correct these so that the labeling is visible. What do thaw 1 and thaw 6 represent? Please mention in the figure legends.
17. Please include a paragraph on the limitations of the Protocol in the Discussion section
18. Please do not abbreviate journal names in references.

Reviewer Comments
Reviewer #1:
Review for Pace et al. JoVE63190

Manuscript summary:
Pace et al describe their scalable production system to make full-length HTT, the Huntington's disease protein, in a mammalian expression system. Further to the protocol, Pace and colleagues describe methods to validate and characterise the samples produced by this protocol. Overall, this manuscript is very well written and the method is detailed and well contextualised. Some minor details are missing from the protocol which should be included. Further data is required to assert the claim regarding native PTMs - see below.

Minor Comments:
Lines 66-69 - Higher resolution structures of the HTT-HAP40 complex are now available in the public domain and these should be referenced: 7DXK and 7DXH - Huang et al (2021) Structure, 6X9O - Harding et al (2021) BioRxiv

New references added: 7DXK and 7DXH - Huang et al (2021) Structure, 6X9O - Harding et al (2021) BioRVix

Line 102 - Not clear what you mean? Do you mean request the gene synthesis service to generate the codon-optimised cDNA? Please clarify the language. This is likely a very expensive option which could be prohibitive to many readers of the protocol, so you may wish to offer an alternative e.g. existing cDNA library clones which might be ordered.

Language clarified. We mainly focused on describing the method we used in the study. Cloning full length HTT cDNA library was not validated in our lab and can require extensive technical development. The plasmids constructed were deposited to the HD Community Repository at the Coriell Institute (www.coriell.org/1/CHDI) and are available free for other researcher to use if the cost is a concern.

Line 110 - More details on this process and Q-length validation would strengthen this section. Currently very brief but this is a key part of the protocol and aim of the protocol - to make different Q-length HTT samples.

Additional information was added. We used a commercial service to synthesize the polyQ region of the DNA and cloned them in the HTT backbone to alter the polyQ length. All transcription regions in the

Line 120 - Anticipated yields? A260/280 and A260/230 ratios?

Line 131 - Great to see the plasmids are being shared. It would be good to see reference to the availability of these reagents in the abstract and/or introduction as the cloning step is probably one of the largest hurdles for users of the protocol.

Line 151 - Anticipated yields? A260/280 and A260/230 ratios?

Line 182 - This section essentially allows validation of expression and determination of expected yields so I think the title should better reflect that aim for this part of the protocol or there should be an additional note more clearly outlining this aim.

Line 257 - What volume aliquots are frozen?

Sections 7-10 - These are the validation/characterisation steps. It could be more helpful to amend the titles to describe the role each of the experiments plays in this process e.g. "Analytical HPLC SEC-MALS-dRI to analyse oligomerization and polydispersity", "Coomassie and Silver Stain to determine sample purity" etc.

Line 377 - Are you referring to FLAG resin reuse? If so, can you comment on the lifetime of your FLAG column following multiple cycles of purification/regeneration? This would be helpful to users of the protocol.

Line 411 - Could it also be that the HTT protein sample is unstable in the gel loading buffer and so it aggregates/associates into higher order oligomers pre-migration?

In our hands, detergent is required to stabilize the monomeric form of FL HTT at 1 mg/mL concentration. Gel loading buffer and run conditions in traditional Native PAGE does not contain detergent. The lack of detergent and the conditions of traditional Native PAGE contribute the observed oligomerization of FL HTT. Coomassie G250 functions as a "mild detergent" in Blue Native PAGE to prevent aggregation of FL HTT.

Native PAGE protocol was removed in the protocol as it is not relevant to FL HTT QC.

Line 425 - Surely the large and disordered regions of structure may also influence gel migration? They would likely increase the hydrodynamic radius of the protein significantly. Additionally, it would appear from the BN-PAGE data that even very small changes in the Q-length influence the migration so is the polyglutamine tract another factor? Comparing migration of Q23 vs Q73 for example the difference in apparent MW is much larger than just the 50 additional residues of the latter construct.

Because ΔExon1-HTT migrates similarly to other polyQ length HTT, instead of N-terminal disordered region, the hollow structure could play a major role in the discrepancy of the migration distance vs MW in Blue Native PAGE.

Materials table - The HEK293 cell line used for production is missing? Similarly, I think the E. coli strain used for large scale plasmid production is also missing.

More information is added to material table. There was confusion what commercial information can be included in the protocol.

Major comments:
Lines 79-81 - How do you know your HEK293-expressed HTT samples have native PTMs? HEK cells have a highly complex karyotype and do not necessarily "function" like physiological human cells. Additional characterisation of the samples produced by mass spectrometry or PTM motif specific antibodies (e.g. those available via the Coriell HD repository for example) would help support this claim.

Language revised. We performed PTM analysis for purified FL HTT and observed similar PTM profile as previous researchers. Data are not included in this manuscript because we are focusing only on the protocol. PTMs are not being compared to any previous publication(s).

Reviewer #2:
The authors describe a method for production of full-length normal and mutant Huntingtin (HTT) following transient transfection with plasmid DNA of human 293 cells grown in suspension culture. The method is scalable and yields are 1-2mg/L cell culture.
The authors did an excellent job with the purification and characterization of the HTT variants, from the first purification steps to the final characterization of the frozen aliquots. There is no doubt that the authors can provide high quality and well characterized material to the HD community.

Minor comments:

1. Line 62: adenovirus (one word)

Revised according to reviewer's suggestion

2. Line 58: the referencing can be improved, they want to cite papers that used insect cells and they cited here Seong at el 2009 (OK) and Huang Bin et al 2015 (that did not use insect cells), instead of Huang Bin et al. 2015 they should rather cite Li et al. 2006 (comes later in the discussion as reference 52).

Revised according to reviewer's suggestion

Also it is suggested to cite the Huang et al. paper in Structure 2021, 29, 804-809, since it is closely related to the author's work.

Revised according to reviewer's suggestion

3. Line 70: adapted instead of adopted

Revised according to reviewer's suggestion

4. Line 90: performed instead of perform

Revised according to reviewer's suggestion

5. Line 107: I assume introduction of the HindIII sites does not change the protein sequence. This should be stated. Will the DNA sequence of the construct be deposited in a public database? This is strongly recommended.

Revised according to reviewer's suggestion; All plasmids were deposited in CHDI for publics to use (see introduction).

6. Lines 141-145: Description of the harvesting and processing of the plasmid DNA from bacteria is very superficial, not really useful. Either remove and refer to Qiagen protocol or describe in more detail.

Reference to Qiagen kit added. It wasn't clear whether a reference to commercial product is allowed in JoVe.

7. Line 155: adjust pH with what?

NaOH added

8. Line 176: what is meant with "add final concentrations of 2 mM butyrate?"

Revised

9. Line 209: use "transfected" instead of "transformed"

10. Line 351: Pellets estimated at ≥ 2 pg??

11. Table of Materials: please organize the table in a way that it is more useful. It appears to be a more or less random summary of materials without any recognizable order, difficult to find anything.
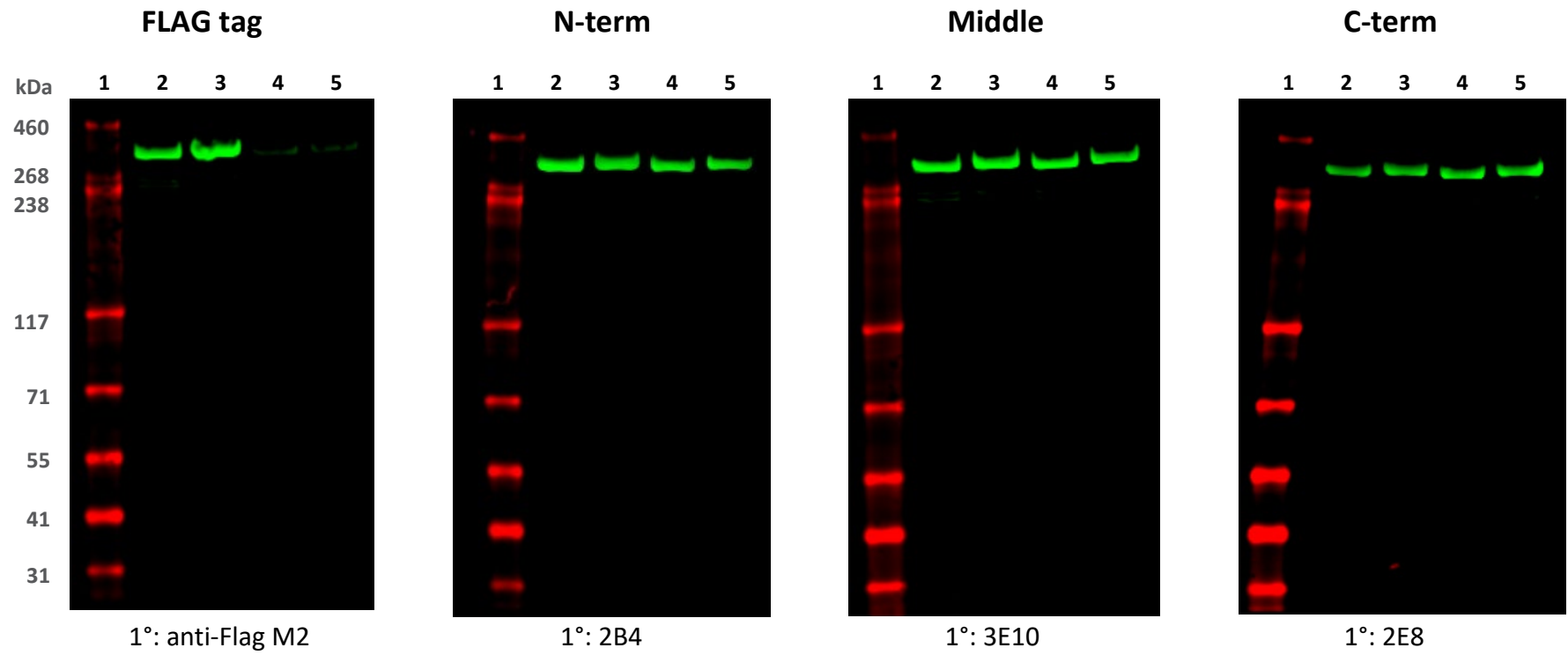
Figure S1

Figure S2

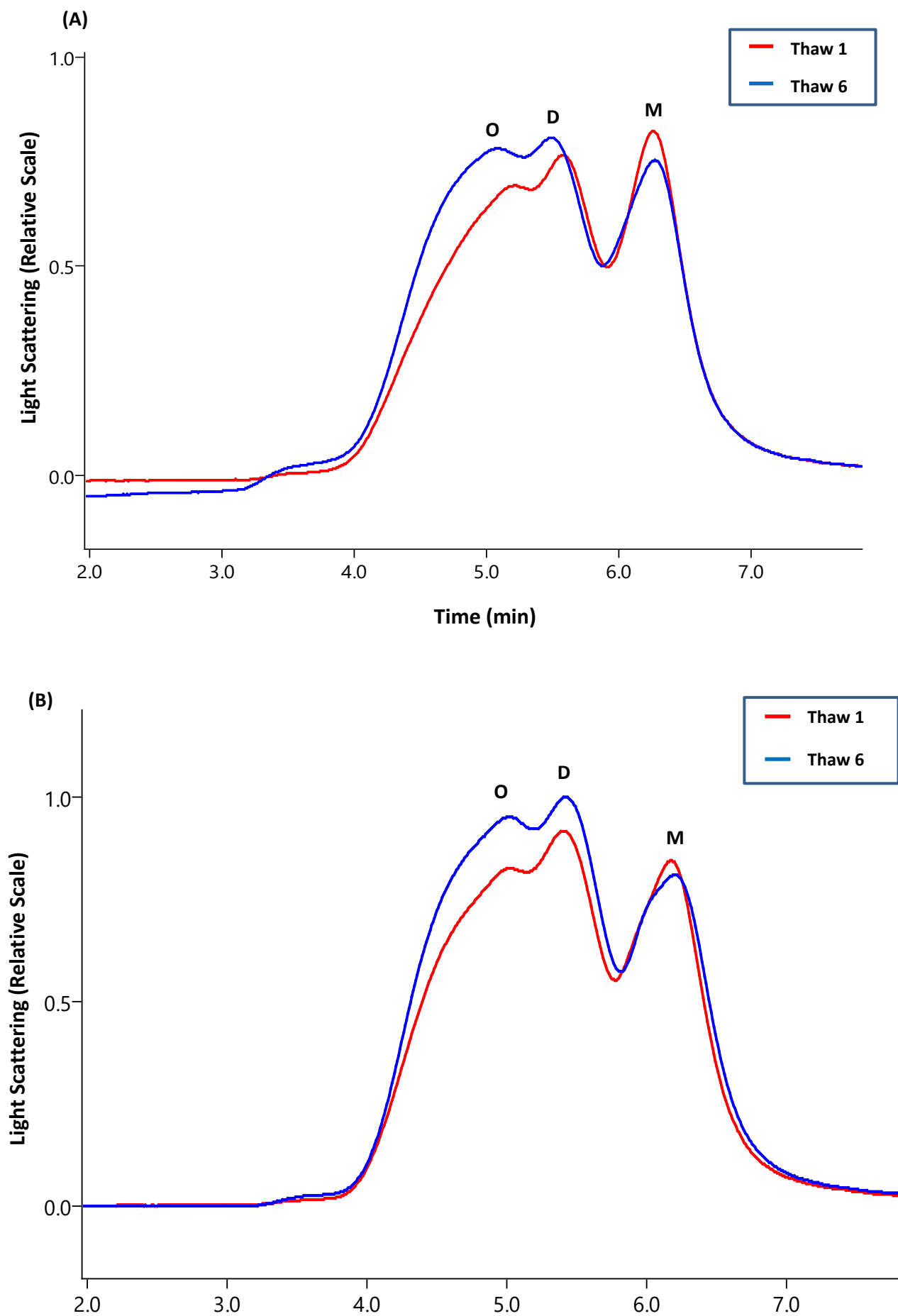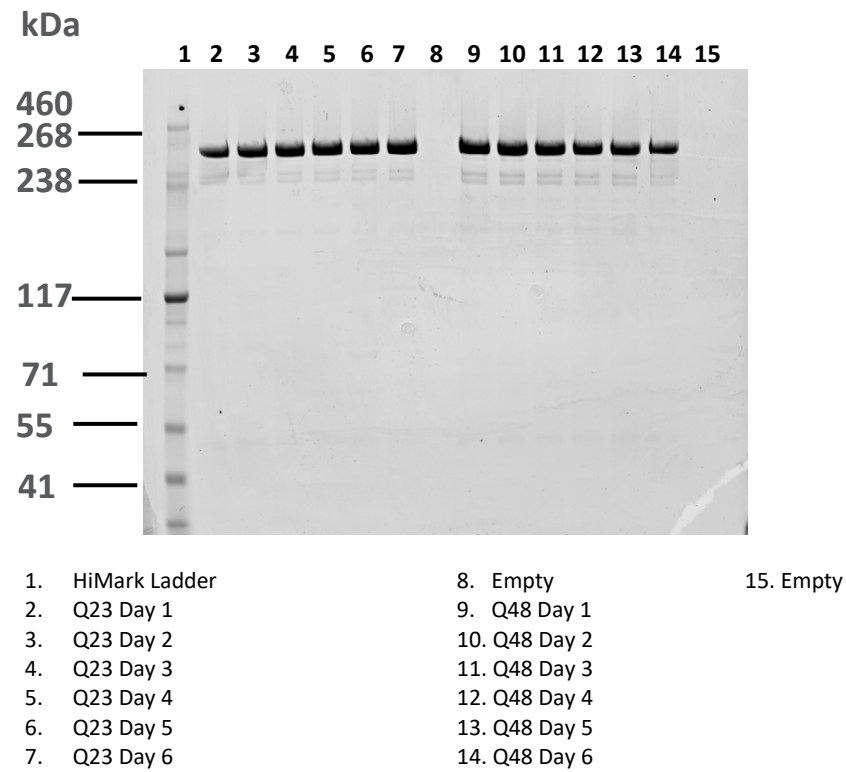**Figure S3**

1. HiMark Ladder
2. Q23 Day 1
3. Q23 Day 2
4. Q23 Day 3
5. Q23 Day 4
6. Q23 Day 5
7. Q23 Day 6

8. Empty
9. Q48 Day 1
10. Q48 Day 2
11. Q48 Day 3
12. Q48 Day 4
13. Q48 Day 5
14. Q48 Day 6

15. Empty

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Supplemental Coding Files
FPLC 15mL AntiFLAG HTT script.xls

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Supplemental Coding Files
FPLC Superose6_26-70 _HTT script.xls