Project:
Biophysical investigation of purified HTT protein samples

Experiment:
Expression and purification of HTT$^{79\text{-}3144}$ with and without HAP40 from Sf9 Insect cells

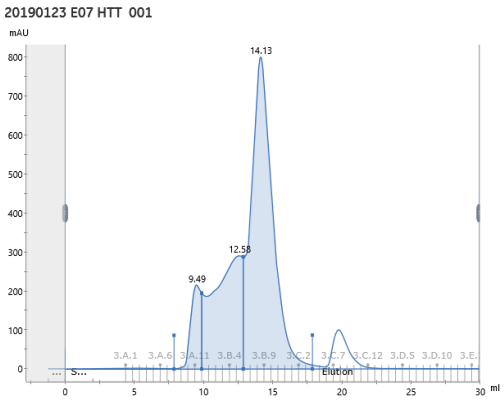
Date completed:
2019/01/28

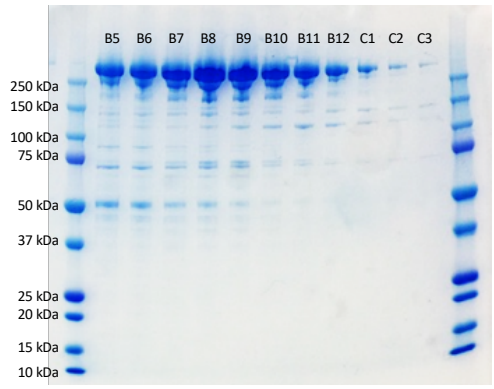
Rationale:
HTT lacking the unstructured region of exon1 (aa. 1-78 which are not resolved in the cryoEM model PDB ID 6EZ8) may serve as a useful control when determining exon1-dependent functionality of the HTT protein. Apo and HAP40-bound HTT$^{79\text{-}3144}$ will be produced from Sf9 insect cell expression system.

Experimental approach:
HTT cDNA spanning residues 79-3144 (assuming Q23) was subcloned into pBacMam2-Di-Ex-LIC and the clone sequence verified. All cloning experiments were performed by Peter Loppnau.

1. **Production of HTT$^{79\text{-}3144}$ TOC017-E07 pBacMam2-Di-Ex-LIC**

Growth: HTT$^{79\text{-}3144}$ (FLAG and His tagged, TOC017-E07) virus used for 8 L BVES sf9 production. Cells were harvested by centrifugation at 4000 rpm, 10 mins, 4 °C (Beckman JLA 8.1000). HTT cell pellets were resuspended in ~320 mL of 20 mM Hepes pH 7.4, 300 mM NaCl, 2.5 % glycerol. Cell resuspensions were spiked with protease inhibitor mix and then stored at -80 ˚C prior to purification in 40 mL aliquots. Full for BVES production methods are here: https://zenodo.org/record/154611. All production experiments were completed by Alma Seitova and Ashley Hutchinson.





Peak	Peak description	Retention (ml)	Area (ml*mAU)	Area %	Ext coeff. (mg ml $^{-1}$ cm $^{-1}$)	Fraction(s)	Volume (ml)	Amount (mg)	Concentration (mg/ml)	Conductivity (mS/cm)
Peak A	Void	9.489	182.9	7.94	0.800	3.A.8 - 3.A.11	2.000	1.143	0.571	27.49
Peak B	Dimer and aggregate species	12.576	701.8	30.49	0.800	3.A.12 - 3.B.5	3.000	4.386	1.462	27.43
Peak C	Monomer	14.134	1417	61.57	0.800	3.B.6 - 3.C.3	5.001	8.858	1.771	27.43

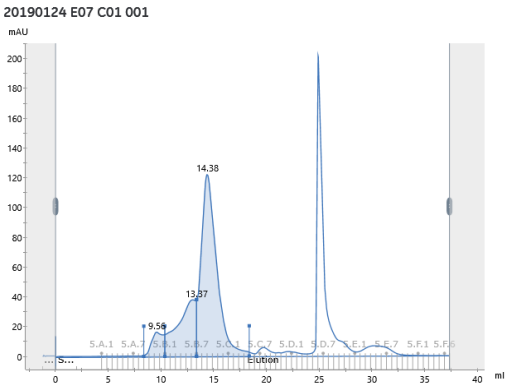
Purification: Cell pastes were thawed and diluted to 1000 mL with 20 mM Hepes pH 7.4, 300 mM NaCl, 2.5 % glycerol and supplemented with benzonase. NB: freeze-thaw cycle for cells is sufficient for lysis. Lysates were clarified by centrifugation at 15,000 rpm, 1 h, 4 °C (Beckman JLA16.2500) and then

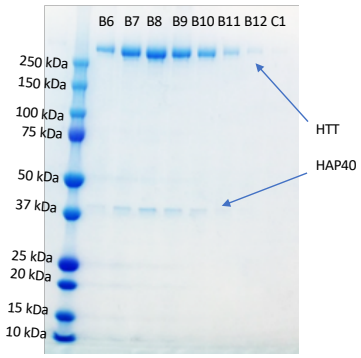
bound to 10 mL anti-FLAG resin (Sigma M2) at 4 ˚C with rocking for 2 hours (flow through – FT1). Lysate-resin mix was transferred to open columns (BioRad) and the resin then washed with 2 x 500 mL 20 mM Hepes pH 7.4, 300 mM NaCl, 2.5 % glycerol (wash – W1 and W2). HTT protein was eluted with ~16 mL resuspension buffer supplemented with 250 µg/mL 3xFLAG peptide (elution – E1). The elution was concentrated to 1 mL (elution concentrated – EC) and run on Superose6 column in 20 mM Hepes pH 7.4, 300 mM NaCl, 1 mM TCEP, 5 % (v/v) glycerol. The monomer peak (~fractions B6-C3) was concentrated then aliquoted and flash frozen in $N_{2\,(l)}$. Samples were analysed by 4-20 % tris-glycine SDS-PAGE.

Yield: 12 x 50 µL, 12 x 20 µL, 14 x 10 µL, 13 x 5 µL @ 7.8 mg/mL -> 8.1 mg total yield

2. Production of HTT$^{79\text{-}3144}$-HAP40: TOC017-E07+TOC011-C01

Growth: 3:1 HTT$^{79\text{-}3144}$ (FLAG and His tagged, TOC017-E07) to HAP40 (His-tagged, TOC011-C01) virus ratios used for 12 L BVES sf9 production. Cells were harvested by centrifugation at 4000 rpm, 10 mins, 4 °C (Beckman JLA 8.1000). HTT cell pellets were resuspended in ~500 mL of 20 mM Hepes pH 7.4, 300 mM NaCl, 2.5 % glycerol. Cell resuspensions were spiked with protease inhibitor mix and then stored at -80 ˚C prior to purification in ~40 mL aliquots. Full for BVES production methods are here: https://zenodo.org/record/154611. All production experiments were completed by Alma Seitova and Ashley Hutchinson.





Peak	Peak description	Retention (ml)	Area (ml*mAU)	Area %	Ext coeff. (mg ml $^{-1}$ cm $^{-1}$)	Fraction(s)	Volume (ml)	Amount (mg)	Concentration (mg/ml)	Conductivity (mS/cm)
Peak A	Void	9.562	20.86	6.84	0.800	5.A.9 - 5.A.12	2.000	0.130	0.065	27.57
Peak B	Dimer and aggregate species	13.371	76.55	25.10	0.800	5.B.1 - 5.B.6	3.001	0.478	0.159	27.57
Peak C	Monomer	14.382	207.6	68.06	0.800	5.B.7 - 5.C.4	5.001	1.297	0.259	27.57

Purification: Cell pastes were thawed and diluted to 1500 mL with 20 mM Hepes pH 7.4, 300 mM NaCl, 2.5 % glycerol and supplemented with benzonase. NB: freeze-thaw cycle for cells is sufficient for lysis. Lysates were clarified by centrifugation at 15,000 rpm, 1 h, 4 °C (Beckman JLA16.2500) and then bound to 10 mL anti-FLAG resin (Sigma M2) at 4 ˚C with rocking for 2 hours (flow through – FT1). Lysate-resin mix was transferred to open columns (BioRad) and the resin then washed with 2 x 500 mL 20 mM Hepes pH 7.4, 300 mM NaCl, 2.5 % glycerol (wash – W1 and W2). HTT protein was eluted with ~16 mL resuspension buffer supplemented with 250 µg/mL 3xFLAG peptide (elution – E1). The sample was then rocked with 3 mL Ni-NTA at 4 ˚C with rocking for 30 mins (flow through – FT2). Ni-NTA beads were washed with 20 mM Hepes pH 7.4, 300 mM NaCl, 2.5 % glycerol then 20 mM Hepes

pH 7.4, 300 mM NaCl, 2.5 % glycerol supplemented with 15 mM imidazole (wash – W3 and W4) and then eluted with 20 mM Hepes pH 7.4, 300 mM NaCl, 2.5 % glycerol, 300 mM imidazole (elution – E3). The elution was concentrated to 1 mL (elution concentrated – EC) and run on Superose6 column in 20 mM Hepes pH 7.4, 300 mM NaCl, 1 mM TCEP, 5 % (v/v) glycerol. The monomer peak (~fractions B7-C2) was concentrated then aliquoted and flash frozen in $N_{2\,(l)}$. Samples were analysed by 4-20 % tris-glycine SDS-PAGE.

Yield: 6 x 20 µL, 8 x 10 µL, 9 x 5 µL @ 4 mg/mL -> 0.98 mg

Conclusions:

$HTT^{79-3144}$ has been cloned into pBacMam2-DiEx-LIC and expressed and purified this protein in its apo form and in complex with HAP40 as we previously described. Both proteins expressed well and the exon1 deficient apo HTT gave extremely high yields – more than 1 mg/L when measured after monomer peak concentration prior to flash freezing. However, there does seem to be clear degradation for this apo sample due to the ladder of bands on the SDS-PAGE, not seen for the HAP40 bound samples. This could be more visible for this sample as the gel is overloaded.

Next steps:

These samples will be used in future protein-protein interaction studies, biophysical analyses and other functional assays with HTT protein as control samples to determine exon1 dependency of HTT functionality.