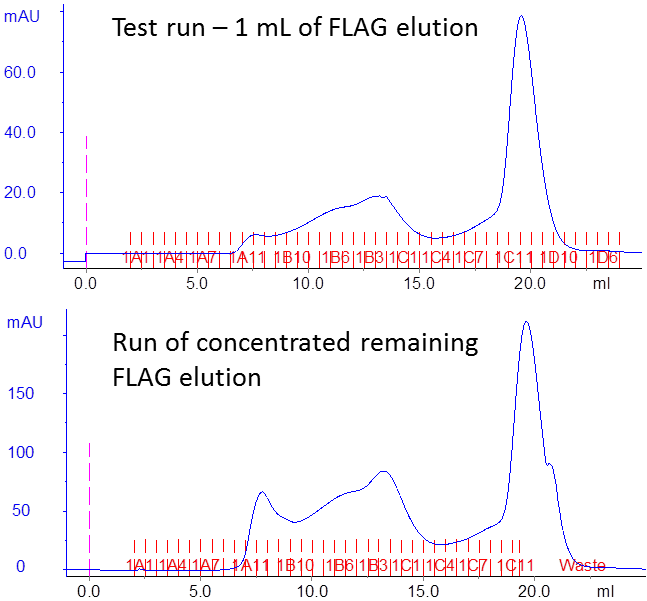
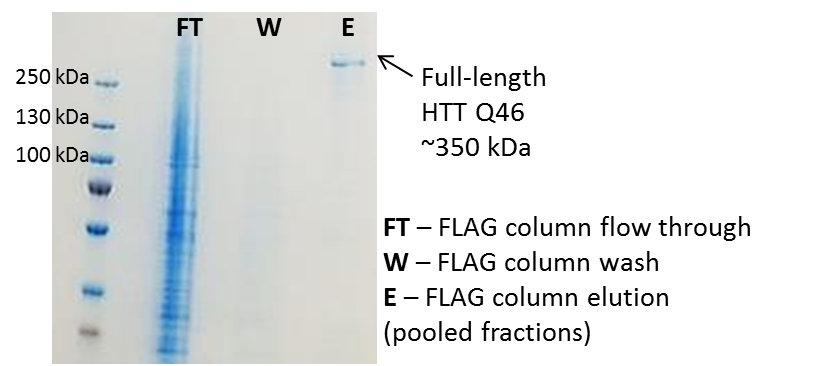
**Huntingtin Q46 expression and purification and optimization of Grafix procedure (2016/09/11)**

Purification as optimised previously:

* 2 L of cells harvested – flash frozen in 12 x 50 mL aliquots in 50 mM Tris-HCl pH8, 500 mM NaCl, 1x PIs
* 2 aliquots freeze-thawed x 2 to lyse. Resuspended in chilled 500 ml 50 mM Tris-HCl pH8, 500 mM NaCl, 1x PIs with benzonase.
* Centrifuge at 15,000 rpm, 1 hour, 4 °C for 1 hour (JA 16.25).
* Incubate clarified lysate each with 1ml equilibrated anti-FLAG M2 resin (Sigma) at 4 °C with rocking for 2 hours in glass bottles.
* Lysate-bead mix loaded into open column and then washed with 1000 ml of 50 mM Tris-HCl pH8, 500 mM NaCl.
* Huntingtin eluted with 2 x 5 ml 400 µg/ml FLAG peptide in 50 mM Tris-HCl pH8, 500 mM NaCl. Incubate beads with peptide for 5 minutes before eluting. (Bradford assay showed no more protein was eluting).
* Run 1ml of elution on Superose6 equilibrated in 20 mM Hepes pH7.4, 300 mM NaCl.

Comment: Previously run in 50 mM Tris-HCl pH8, 500 mM NaCl, need to verify protein does not precipitate/aggregate in these conditions. Monomer peak still visible in UV trace.

* Concentrate remaining sample and rerun column.

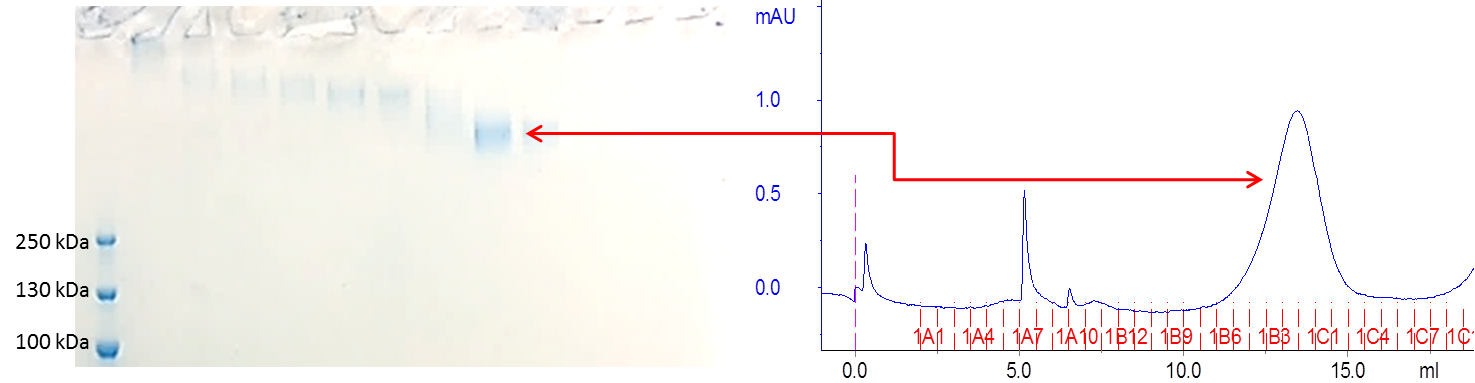


Small-scale optimization of Grafix procedure:

* Gradients poured with 5-20 % (w/v) sucrose and 0-0.2 % (v/v) glutaraldehyde in 20 mM HEPES pH7.4, 300 mM NaCl. 2 continuous gradients poured using gradient mixer and 2 x 5-layer gradient (5+0, 8+0.05, 12+0.1, 16+0.15, 20+0.2 % sucrose+glutaraldehyde) layered by hand pipetting. All 8 mL total volume.
* To each type of gradient poured either 30 or 60 µg of huntingtin protein from the monomeric peak was applied. Samples centrifuged at 108,000 *xg* for 18 hours.
* Tubes pierced on bottom and fractionated into ~500 µL aliquots.
* Bradford/BCA/nanodrop/spectrophotometer all tried and failed to identify fractions with protein in – sucrose and glutaraldehyde interfere with assays and A280 readings.
* Gel samples (~28 µL of protein per well) run on 4-20% Tris-glycine SDS-PAGE (wedge-well) and stained with Blue-Silver Coomassie stain overnight, destained in water.
* Bands visible showing different oligomeric species – stable on gel due to chemical cross linking.
* Fraction with HTT of lowest MW run on Superose 6 – runs at monomer peak position, elution volume ~13.5 mL.

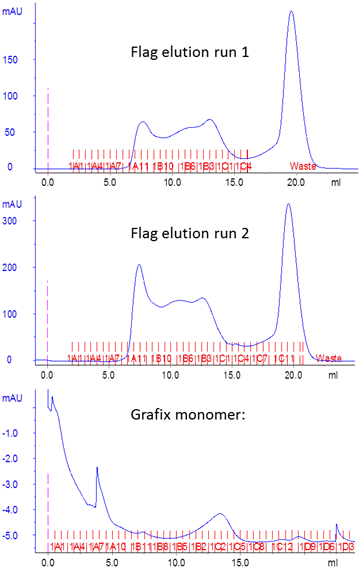
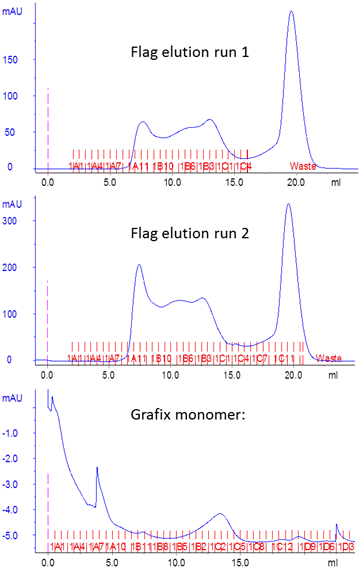
Comment: Method works but not enough protein sample. No real difference between gradients. Continuous gradient more reproducible and easier to set up so use in future experiments. Amount of sample applied does not seem to affect oligomer distribution so load largest amount i.e. 60 µg per 8 mL.

20/0.2% Sucrose/glutaraldehyde gradient 5/0%

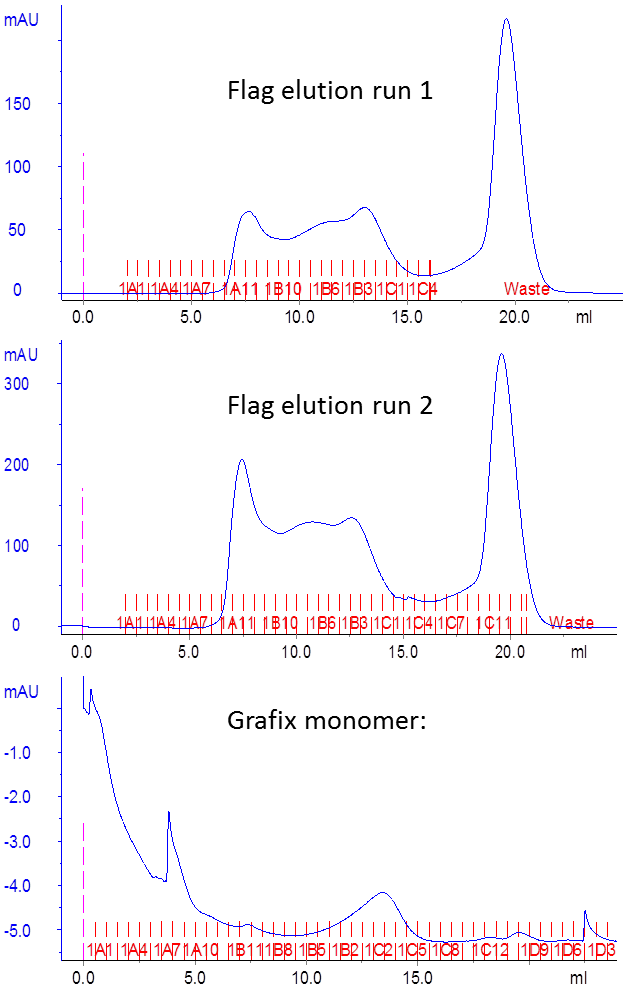


Scaled up purification/Grafix:

* 8 L of cells purified with appropriately scaled up protocol
* Superose6 GF split into 2 runs – 1st run monomer peak concentrated to 2 mg/mL, aliquoted to 20 µL and flash frozen – 12 aliquots. 2nd run monomer peak applied to Grafix procedure.

* Grafix used 6 x 30 mL gradients poured using gradient mixer, 250 µg of protein applied to each. Centrifuge at 108,000 xg for 16 hours. Fractionate into 1.6 mL fractions ~19-20 fractions per tube.
* Gel samples (~28 µL of protein per well) run on 4-20% Tris-glycine SDS-PAGE (wedge-well) and stained with Blue-Silver Coomassie stain overnight, destained in water.
* Run gel of last 14 fractions from each tube to pick out monomer fractions, buffer exchange, concentrate and freeze: 4 x 20 µL aliquots at 0.5 mg/mL.
* Remaining monomer fraction sample run on Superose6 to confirm monomer elution position.

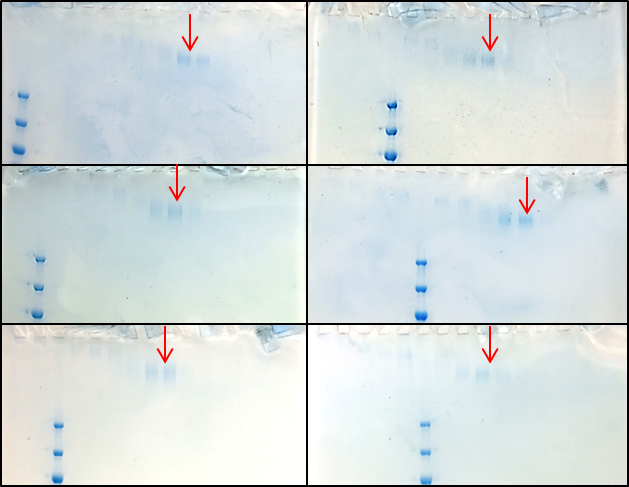
 

Comments, amendments and follow up for Huntingtin EM sample preparation:

* Approximately consistent position of monomer 7th, 8th or 9th gel sample, corresponding to 13th, 14th or 15th fraction (~0.05-0.08 (v/v) glutaraldehyde and 8-10 (w/v) % sucrose) out of 20 fractions. Next time, just these fractions could be run on SDS-PAGE to verify presence of monomer.

Sucrose/glutaraldehyde gradient

20/0.2% 5/0% 20/0.2% 5/0%

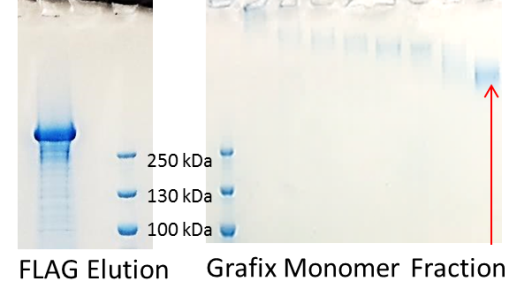


Tube 1 Tube 4

Tube 2 Tube 5

Tube 3 Tube 6

* Although sample can be run on Superose6 GF in 20 mM HEPES pH 7.4, 300 mM NaCl, reduced salt could increase the ratio of monomer:aggregate peak and reduce overall amounts of protein for Grafix step. Keep [NaCl] at ~ 0.5 M until final buffer exchange. No need to drop during Grafix procedure and should increase yield of monomer.
* Concern as smallest HTT species on gel runs larger than un-Grafix modified sample and smeary but likely a result of cross linking. Bands appear smeary as crosslinking is not uniform across all particles so different points in molecule linked together therefore retarding different on the gel. Protein runs higher on gel also due to crosslinking as no longer linear polymer on denatured gel.



* 3 x 20 µL HTT Q46 1-3144 @ 2 mg/mL (from Superose6 GF elution, monomer fraction, non-Grafix treated) and 3 x 20 µL HTT Q 46 1-3144 @ 0.5 mg/mL (Grafix treated monomer fraction) shipped to Prof. Susan Lea, arrived Friday 16th September. Awaiting validation from Susan regarding grid set up.