**Project:**
High resolution structural analysis of purified HTT samples

**Experiment:**
Large-scale purification of HTT domain constructs

**Date completed:**
2019/04/01

**Rationale:**
Domain fragments of the huntingtin protein are useful reagents for examining protein-protein interaction characteristics of huntingtin and to map interaction interfaces. Stable, monodisperse and pure samples may also be amenable to high resolution structure solution by X-ray crystallography.
Previously huntingtin fragments were cloned and screened for expression in small-scale (3 mL) culture experiments. Positive hits were scaled for production as detailed in the table on the next page. All previous work is described in this post: https://zenodo.org/record/2600051#.XKU89aeZPOQ.

| Construct ID | Construct AA start | Construct AA end | Pipeline Position | Batch and Plate Well | Corresponding Domain(s) | Tag | Follow up production |
|---|---|---|---|---|---|---|---|
| HTT_76_404-pBacMam2-DiEx-LIC | 76 | 404 | Cloned | TOC019A01 | N-HEAT 1 | …AENLYFQSHHHHHHHHHHHDYKDDDDK | TestX |
| HTT_81_404-pBacMam2-DiEx-LIC | 81 | 404 | Cloned | TOC019A02 | N-HEAT 1 | …AENLYFQSHHHHHHHHHHHDYKDDDDK | 100 mL |
| HTT_93_404-pBacMam2-DiEx-LIC | 93 | 404 | Cloned | TOC019A03 | N-HEAT 1 | …AENLYFQSHHHHHHHHHHHDYKDDDDK | 4 L |
| HTT_663_1643-pBacMam2-DiEx-LIC | 663 | 1643 | Cloned | TOC019A04 | N-HEAT 2 | …AENLYFQSHHHHHHHHHHHDYKDDDDK | TestX |
| HTT_663_1715-pBacMam2-DiEx-LIC | 663 | 1715 | Cloned | TOC019A05 | N-HEAT 2 | …AENLYFQSHHHHHHHHHHHDYKDDDDK | TestX |
| HTT_76_1643-pBacMam2-DiEx-LIC | 76 | 1643 | Cloned | TOC019A06 | N-HEAT 1 + 2 | …AENLYFQSHHHHHHHHHHHDYKDDDDK | 100 mL |
| HTT_81_1643-pBacMam2-DiEx-LIC | 81 | 1643 | Cloned | TOC019A07 | N-HEAT 1 + 2 | …AENLYFQSHHHHHHHHHHHDYKDDDDK | 100 mL |
| HTT_76_1715-pBacMam2-DiEx-LIC | 76 | 1715 | Cloned | TOC019A08 | N-HEAT 1 + 2 | …AENLYFQSHHHHHHHHHHHDYKDDDDK | 100 mL |
| HTT_81_1715-pBacMam2-DiEx-LIC | 81 | 1715 | Cloning Failed | TOC019A09 | N-HEAT 1 + 2 | …AENLYFQSHHHHHHHHHHHDYKDDDDK | N/A |
| HTT_1731_2064-pBacMam2-DiEx-LIC | 1731 | 2064 | Cloned | TOC019A10 | BRIDGE | …AENLYFQSHHHHHHHHHHHDYKDDDDK | TestX |
| HTT_2088_3100-pBacMam2-DiEx-LIC | 2088 | 3100 | Cloned | TOC019A11 | C-HEAT | …AENLYFQSHHHHHHHHHHHDYKDDDDK | 100 mL |
| HTT_2088_3144-pBacMam2-DiEx-LIC | 2088 | 3144 | Cloned | TOC019A12 | C-HEAT | …AENLYFQSHHHHHHHHHHHDYKDDDDK | 100 mL |
| HTT_2094_3100-pBacMam2-DiEx-LIC | 2094 | 3100 | Cloned | TOC019B01 | C-HEAT | …AENLYFQSHHHHHHHHHHHDYKDDDDK | TestX |
| HTT_76_404-pBacMam2-DiEx-LIC | 76 | 404 | Cloned | TOC019B02 | N-HEAT 1 | …DYKDDDDK | 4 L |
| HTT_81_404-pBacMam2-DiEx-LIC | 81 | 404 | Cloned | TOC019B03 | N-HEAT 1 | …DYKDDDDK | 4 L |
| HTT_93_404-pBacMam2-DiEx-LIC | 93 | 404 | Cloned | TOC019B04 | N-HEAT 1 | …DYKDDDDK | 100 mL |
| HTT_663_1643-pBacMam2-DiEx-LIC | 663 | 1643 | Cloned | TOC019B05 | N-HEAT 2 | …DYKDDDDK | TestX |
| HTT_663_1715-pBacMam2-DiEx-LIC | 663 | 1715 | Cloned | TOC019B06 | N-HEAT 2 | …DYKDDDDK | TestX |
| HTT_76_1643-pBacMam2-DiEx-LIC | 76 | 1643 | Cloned | TOC019B07 | N-HEAT 1 + 2 | …DYKDDDDK | 100 mL |
| HTT_81_1643-pBacMam2-DiEx-LIC | 81 | 1643 | Cloned | TOC019B08 | N-HEAT 1 + 2 | …DYKDDDDK | 4 L |
| HTT_76_1715-pBacMam2-DiEx-LIC | 76 | 1715 | Cloned | TOC019B09 | N-HEAT 1 + 2 | …DYKDDDDK | TestX |
| HTT_81_1715-pBacMam2-DiEx-LIC | 81 | 1715 | Cloning Failed | TOC019B10 | N-HEAT 1 + 2 | …DYKDDDDK | N/A |
| HTT_1731_2064-pBacMam2-DiEx-LIC | 1731 | 2064 | Cloned | TOC019B11 | BRIDGE | …DYKDDDDK | TestX |
| HTT_2088_3100-pBacMam2-DiEx-LIC | 2088 | 3100 | Cloned | TOC019B12 | C-HEAT | …DYKDDDDK | TestX |
| HTT_2088_3144-pBacMam2-DiEx-LIC | 2088 | 3144 | Cloned | TOC019C01 | C-HEAT | …DYKDDDDK | 4 L |
| HTT_2094_3100-pBacMam2-DiEx-LIC | 2094 | 3100 | Cloned | TOC019C02 | C-HEAT | …DYKDDDDK | 100 mL |

*Details of pBacMam2-DiEx-LIC HTT fragment constructs expression profiles in small-scale Sf9 culture. Constructs with bands of the expected size for each construct on SDS-PAGE are highlighted in green for good levels of expression and yellow for those with lower levels of expression.*

**Experimental approach and findings:**

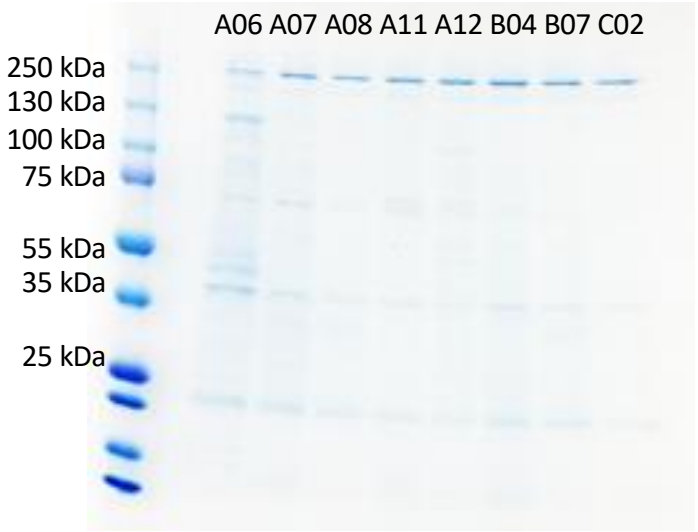
1. **TOC019 Medium scale production – 100 mL**

Constructs:

| Construct ID | Construct AA start | Construct AA end | Batch and Plate Well | Corresponding Domain(s) | Tag |
|---|---|---|---|---|---|
| HTT_76_1643-pBacMam2-DiEx-LIC | 76 | 1643 | TOC019A06 | N-HEAT 1 + 2 | …AENLYFQSHHHHHHHHHHDYKDDDDK |
| HTT_81_1643-pBacMam2-DiEx-LIC | 81 | 1643 | TOC019A07 | N-HEAT 1 + 2 | …AENLYFQSHHHHHHHHHHDYKDDDDK |
| HTT_76_1715-pBacMam2-DiEx-LIC | 76 | 1715 | TOC019A08 | N-HEAT 1 + 2 | …AENLYFQSHHHHHHHHHHDYKDDDDK |
| HTT_2088_3100-pBacMam2-DiEx-LIC | 2088 | 3100 | TOC019A11 | C-HEAT | …AENLYFQSHHHHHHHHHHDYKDDDDK |
| HTT_2088_3144-pBacMam2-DiEx-LIC | 2088 | 3144 | TOC019A12 | C-HEAT | …AENLYFQSHHHHHHHHHHDYKDDDDK |
| HTT_93_404-pBacMam2-DiEx-LIC | 93 | 404 | TOC019B04 | N-HEAT 1 | …DYKDDDDK |
| HTT_76_1643-pBacMam2-DiEx-LIC | 76 | 1643 | TOC019B07 | N-HEAT 1 + 2 | …DYKDDDDK |
| HTT_2094_3100-pBacMam2-DiEx-LIC | 2094 | 3100 | TOC019C02 | C-HEAT | …DYKDDDDK |

Growth:

100 mL of Sf9 cell culture at a density of ~4.5 million cells per mL were infected with 0.2 mL of P3 recombinant baculovirus and grown at 130 rpm and 27 °C. HyQ SFX insect serum medium containing 10 µg/mL gentamicin was used as the culture medium. Infected cells were harvested when viability dropped to 80%–85%, normally after ~72 h post-infection. Cells were harvested by centrifugation and stored at -80 ˚C prior to purification.



Purification:

Cells were thawed and resuspended in 40 mL with 20 mM Hepes pH 7.4, 500 mM NaCl, 5 % glycerol and supplemented with benzonase and protease inhibitors. NB: freeze-thaw cycle for cells is sufficient for lysis. Lysates were clarified by centrifugation at 20,000 rpm, 20 mins, 4 °C (Beckman JA25.50) and then bound to 0.5 mL anti-FLAG resin (Sigma M2) at 4 ˚C with rocking for 2 hours. Lysate-resin mix was transferred to open columns (BioRad) and the resin then washed with 2 x 50 mL 20 mM Hepes pH 7.4, 300 mM NaCl, 2.5 % glycerol. HTT protein was eluted with ~2 mL 0.1 M glycine pH 3. The elution was concentrated to 60 µL. 20 µL 4 x loading dye was added and samples were analysed by 4-20 % tris-glycine SDS-PAGE.

No clear overexpression bands seen for targets. Not to be pursued any further.

## 2. TOC019 Large scale production – 4 L

Constructs:

| Construct ID | Construct AA start | Construct AA end | Batch and Plate Well | Corresponding Domain(s) | Tag |
|---|---|---|---|---|---|
| HTT_81_404-pBacMam2-DiEx-LIC | 81 | 404 | TOC019A02 | N-HEAT 1 | …AENLYFQSHHHHHHHHHHDYKDDDDK |
| HTT_93_404-pBacMam2-DiEx-LIC | 93 | 404 | TOC019A03 | N-HEAT 1 | …AENLYFQSHHHHHHHHHHDYKDDDDK |
| HTT_76_404-pBacMam2-DiEx-LIC | 76 | 404 | TOC019B02 | N-HEAT 1 | …DYKDDDDK |
| HTT_81_404-pBacMam2-DiEx-LIC | 81 | 404 | TOC019B03 | N-HEAT 1 | …DYKDDDDK |
| HTT_81_1643-pBacMam2-DiEx-LIC | 81 | 1643 | TOC019B08 | N-HEAT 1 + 2 | …DYKDDDDK |
| HTT_2088_3144-pBacMam2-DiEx-LIC | 2088 | 3144 | TOC019C01 | C-HEAT | …DYKDDDDK |

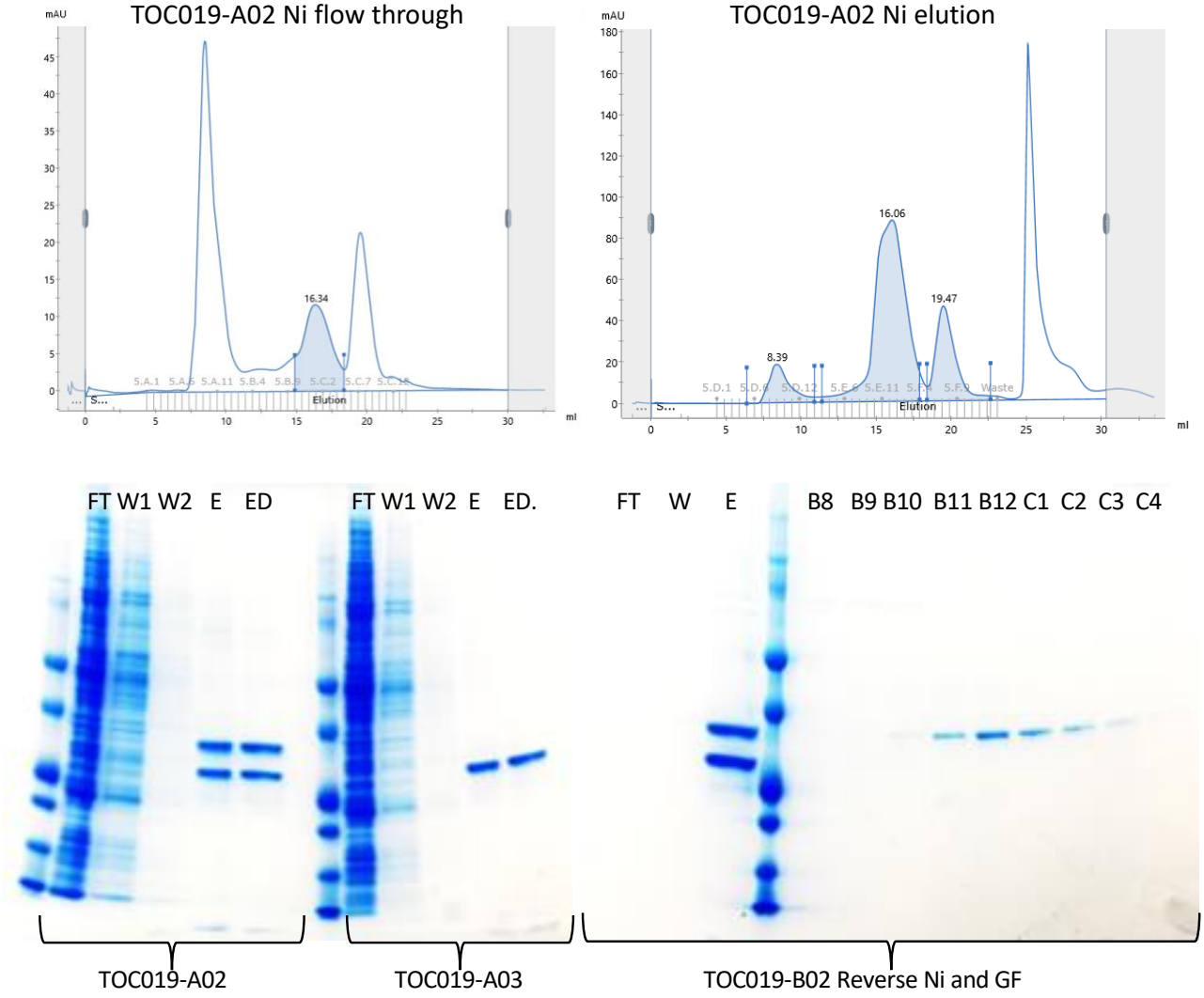
Growth:

4 L of Sf9 cell culture at a density of ~4.5 million cells per mL were infected with 8 mL of P3 recombinant baculovirus and grown at 130 rpm and 27 °C. HyQ SFX insect serum medium containing 10 µg/mL gentamicin was used as the culture medium. Infected cells were harvested when viability dropped to 80%–85%, normally after ~72 h post-infection. Cell resuspensions were spiked with protease inhibitor mix and then stored at -80 ˚C prior to purification in 4 x 40 mL aliquots.

Purification for A02 and A03:

Cell pastes were thawed and diluted to 500 mL with 20 mM Hepes pH 7.4, 500 mM NaCl, 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 5 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 250 mL 20 mM Hepes pH 7.4, 300 mM NaCl, 2.5 % glycerol (wash – W1 and W2). HTT protein was eluted with ~16 mL

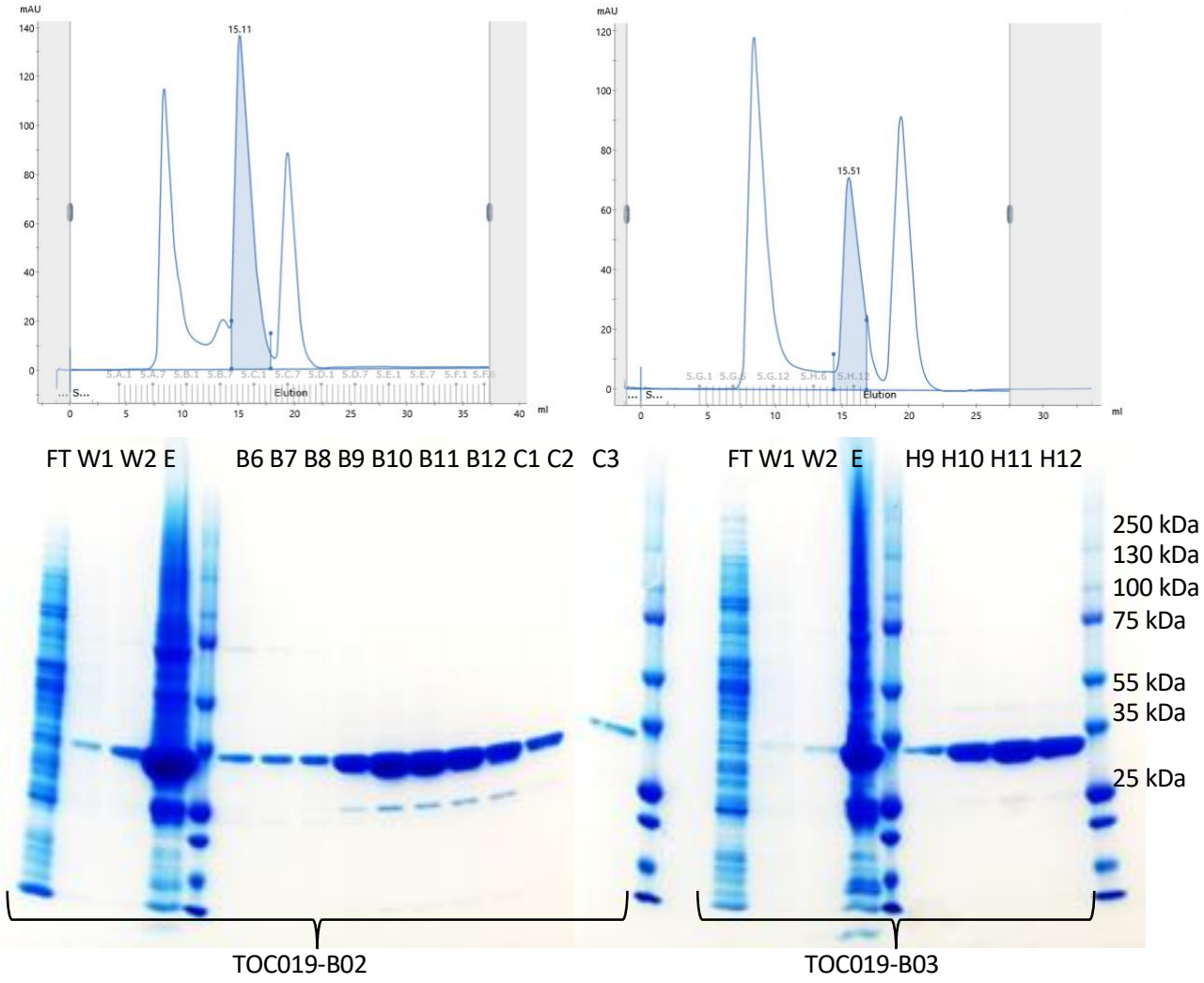
resuspension buffer supplemented with 250 µg/mL 3xFLAG peptide (elution - E). ~1 mg of TEV protease was added and the samples incubated over night at 4 °C. A03 shows no expression so this purification was not continued. A02 elution sample was then rocked with 3 mL Ni-NTA at 4 ˚C with rocking for 30 mins (flow through – FT). 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 – W) and then eluted with 20 mM Hepes pH 7.4, 300 mM NaCl, 2.5 % glycerol, 300 mM imidazole (elution – E). The elution was concentrated to 1 mL (elution concentrated – EC) and run on S200 10/300 GL column in 20 mM Hepes pH 7.4, 300 mM NaCl, 1 mM TCEP, 5 % (v/v) glycerol. Samples were analysed by 4-20 % tris-glycine SDS-PAGE.




Most of A02 was not digested and therefore only a low yield is obtained from the Ni flow through run. Ni elution sample still contains uncleaved A02 and TEV which can not be separated on gel filtration. However, this sample does seem very stable and shows almost no degradation.

Purification for B02 and B03:

Cell pastes were thawed and diluted to 500 mL with 20 mM Hepes pH 7.4, 500 mM NaCl, 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 5 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 250 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 S200 10/300 GL column in 20 mM Hepes pH 7.4, 300 mM NaCl, 1 mM TCEP, 5 % (v/v) glycerol. Samples were analysed by 4-20 % tris-glycine SDS-PAGE.



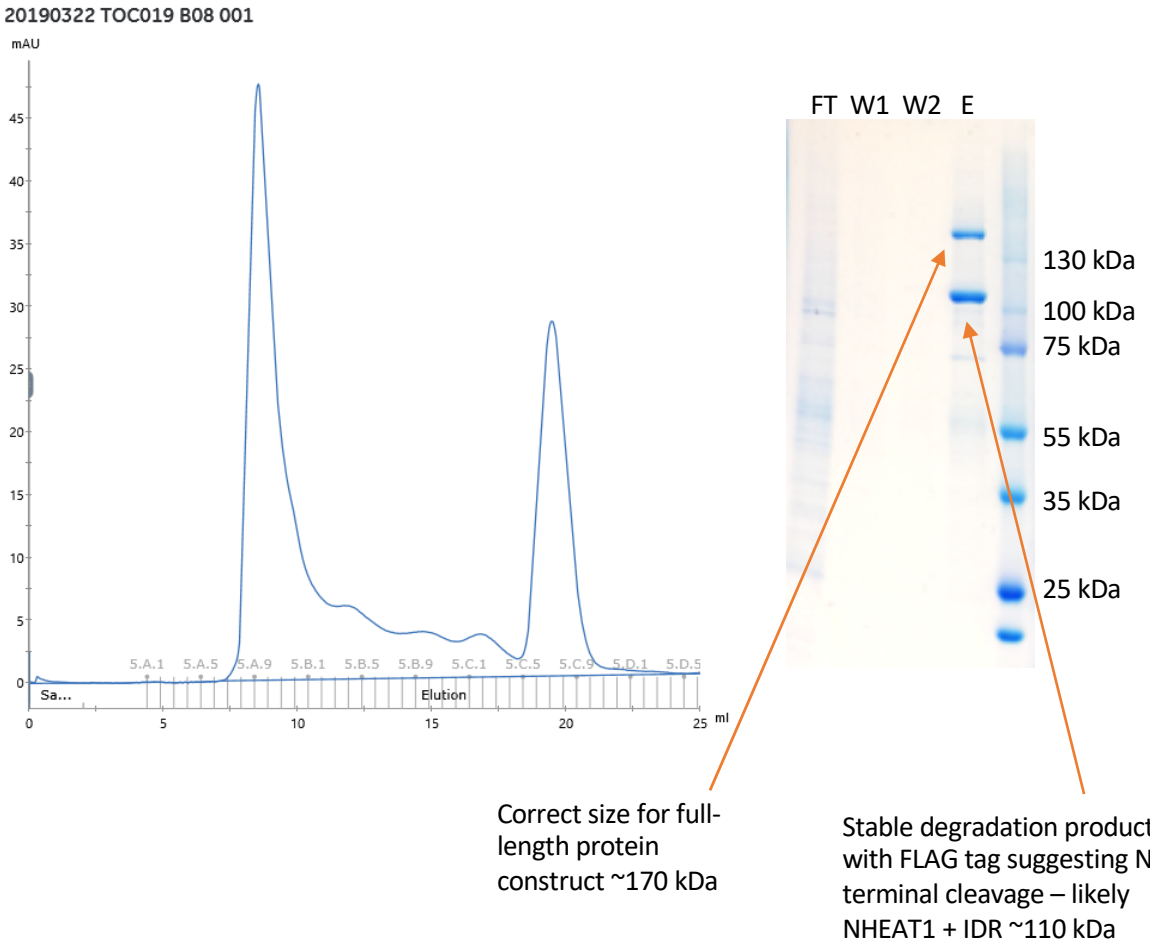

Follow up: Constructs B02 and B03 show good yields, monodisperse peaks and are fairly pure samples. Samples concentrated and set up into crystallisation trays at 298 K. B02 seems to also have a dimer shoulder peak (fractions B6-B8)?

Crystal tray set up completed with Firebird crystallisation robot at room temperature:

TOC019-B02:
7.2 mg/mL, 2 x 1 µL drop at 1:1 & 1:3 protein:well solution, SGC Screen II and Red Wings
5 mg/mL, 1 x 1 µL drop at 1:1 protein:well solution, SGC Screen II and Red Wings
TOC019-B03:
8 mg/mL, 1 x 1 µL drop at 1:1 protein:well solution, SGC Screen II and Red Wings

Purification for B08:

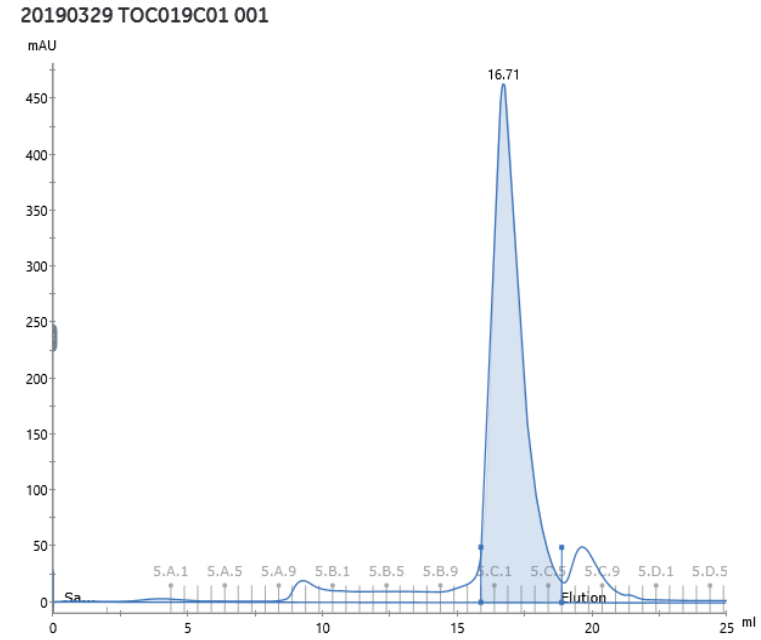
Cell pastes were thawed and diluted to 500 mL with 20 mM Hepes pH 7.4, 500 mM NaCl, 5 % glycerol and supplemented with benzonase. NB: freeze-thaw cycle for cells is sufficient for lysis. Lysates were clarified by centrifugation at 15,000 rpm, 1 h, 4 °C (Beckman JLA16.2500) and then bound to 5 mL anti-FLAG 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 250 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 - E). The elution was concentrated to 1 mL (elution concentrated – EC) and run on S200 10/300 GL column in 20 mM Hepes pH 7.4, 300 mM NaCl, 1 mM TCEP, 5 % (v/v) glycerol. Samples were analysed by 4-20 % tris-glycine SDS-PAGE.



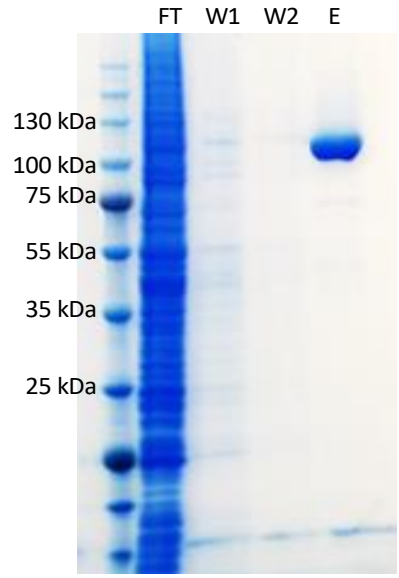

2 bands indicates cleavage to stable product? Sample is eluting in void according to GF profile – need to use Superose6 next time?

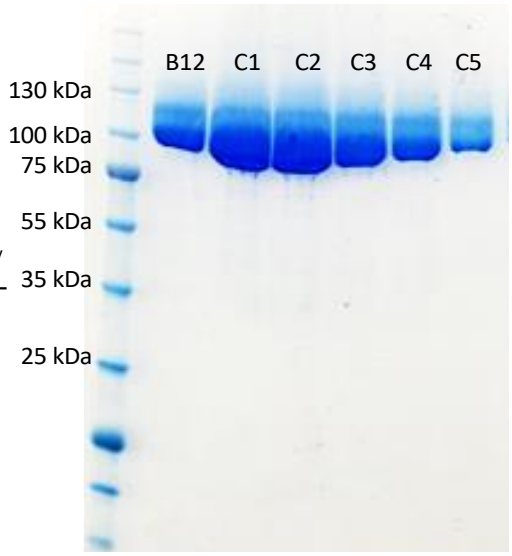
Purification for C01:

Cell pastes were thawed and diluted to 500 mL with 20 mM Hepes pH 7.4, 500 mM NaCl, 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 5 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 250 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 - E). The elution was concentrated to 1 mL (elution concentrated – EC) and run on Superose6 10/300 GL column in 20 mM Hepes pH 7.4, 300 mM NaCl, 1 mM TCEP, 5 % (v/v) glycerol. Samples were analysed by 4-20 % tris-glycine SDS-PAGE.




| Retention (ml) | Area (ml*mAU) | Ext coeff. (mg ml $^{-1}$ cm $^{-1}$) | Fraction(s) | Volume (ml) | Amount (mg) | Concentration (mg/ml) | Conductivity (mS/cm) |
|---|---|---|---|---|---|---|---|
| 16.712 | 580.4 | 1.000 | 5.B.12 - 5.C.5 | 3.001 | 2.902 | 0.967 | 26.21 |




Very clean sample – set up crystal trays: 4 trays - 1:1 drop at 5 mg/mL or 10 mg/mL in Red Wings or SGC Screen. Remaining sample flash frozen – 20 µL x 4, 10 µL x 5 at 7.5 mg/mL.