Protein information sheet

Gene name	**UBQLN2**
Uniprot ID	Q9UHD9
Region	M1 - S624

Description	Ubiquilin 2
Synonyms	ALS15, CHAP1, DSK2, HRIHFB2157, N4BP4, PLIC2.
Construct ID	UBQLN2A-c000
Protein sequence	*SM*AENGESSGPPRPSRGPAAAQGSAAAPAEPKIIKVTVKTPKEKEEFAVPENS SVQQFKEAISKRFKSQTDQLVLIFAGKILKDQDTLIQHGIHDGLTVHLVIKSQ NRPQGQSTQPSNAAGTNTTSASTPRSNSTPISTNSNPFGLGSLGGLAGLSSLG LSSTNFSELQSQMQQQLMASPEMMIQIMENPFVQSMLSNPDLMRQLIMANPQM QQLIQRNPEISHLLNNPDIMRQTLEIARNPAMMQEMMRNQDLALSNLESIPGG YNALRRMYTDIQEPMLNAAQEQFGGNPFASVGSSSSSGEGTQPSRTENRDPLP NPWAPPPATQSSATTSTTTSTGSGSGNSSSNATGNTVAAANYVASIFSTPGMQ SLLQQITENPQLIQNMLSAPYMRSMMQSLSQNPDLAAQMMLNSPLFTANPQLQ EQMRPQLPAFLQQMQNPDTLSAMSNPRAMQALMQIQQGLQTLATEAPGLIPSF TPGVGVGVLGTAIGPVGPVTPIGPIGPIVPFTPIGPIGPIGPTGPAAPPGSTG SGGPTGPTVSSAAPSETTSPTSESGPNQQFIQQMVQALAGANAPQLPNPEVRF QQQLEQLNAMGFLNREANLQALIATGGDINAAIERLLGSQPSS*SKGGYGLNDI* *FEAQKIEWHE*

Protein mass	with biotin, + 226.1	Protein pI	Extinction (M^{-1} cm^{-1})
68174	68400	5.11	18450

Expression host	E. coli BL21(DE3)-R3-BirA
Expression medium	Terrific broth (TB) with 50 µg/ml kanamycin, 50 µg/ml Streptomycin and Chloramphenicol (34 µg/ml) included in starter cultures but not in final growth. Biotin (10 mM stock): Add 24mg of d-biotin to 10 ml of warm (microwaved) 10mM bicine buffer (pH 8.3) and sterilize of the solution with a syringe and a 0.2 micron filter.
Transformation and storage	Cells were transformed with the expression plasmid and selected on LB-agar plate's containing kanamycin, streptomycin and chloramphenicol. Several colonies were picked from the overnight transformation and used to inoculate cultures in LB supplemented with KAN/Cam/Str which were grown overnight and stored as glycerol (15% v/v) stock at -80°C.
Expression and *in vivo* biotinylation	10ml starter cultures in LB supplemented with KAN/Cam/Str were grown overnight at 37°C with shaking at 250rpm. Next day, 10 ml was used to inoculate 1L of TB in a 2LUltraYield flask supplemented with Streptomycin 50µg/ml and Kanamycin 50µg/ml. **Note:** the medium need not contain chloramphenicol at this step; the pRARE2 plasmid is not lost during the growth process and also ensures a more predictable growth. Seal flask

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	with an adhesive air porous film during cell growth. Culture was grown in a 37°C incubator with shaking at 200rpm. Cell growth was monitored until Mid-log phase was reached and cell density OD_{600nm} was approximately 2.00 – 3.00 in TB. Incubator temperature was lowered to 18ºC and culture flask allowed to cool. After 30 - 45 minutes protein expression was induced by the addition of IPTG at 0.1mM final concentration. During the induction 10ml of biotin solution is added to each 1L culture on induction with IPTG. Incubation of culture was continued for 16 hours at 18°C. Next day, the cells were collected by centrifugation (15 min, 5,000 RPM in JLS-8.100 rotors). The supernatant discarded and th(10 mM)e cell pellet transferred into a 50ml conical polypropylene tube. Cell pellet was weighed, frozen and stored at -80°C.
Purification buffers	2x lysis buffer: 100 mm HEPES, pH 7.5, 1.0 M NaCl, 10% v/v glycerol, 1mM TCEP, 10mM imidazole. Protease inhibitors (1:200 Merck cocktail IV) were added before use. 1x lysis buffer: 50 mm HEPES, pH 7.5, 0.5 M NaCl, 10% v/v glycerol, 1 mM TCEP, 10 mM imidazole Wash buffer: 50 mm HEPES, pH 7.5, 0.5 M NaCl, 10% v/v glycerol, 1 mM TCEP, 40 mM imidazole Elution buffer: 50mM Hepes pH 7.5, 500mM NaCl, 10% Glycerol, 1mM TCEP, 100mM, 150mM, 250Mm, 500 Imidazole, respectively GF buffer: 20 mM HEPES, pH 7.5, 0.5M NaCl, 5% v/v glycerol, 1 mM TCEP PEI 5%, pH 7.5: 5% polyethyleneimine (diluted from 50% stock, Sigma) and **titrated to pH 7.5 with HCl.**
Purification step 1: IMAC	**Cell Lysis:** Frozen cell pellets from -80°C were thawed by intermittently placing in a 37°C water bath, then transferred directly to ice. Cells were re-suspended in pre-chilled lysis buffer by vortex or pipetting gentle until cells were completely in suspension. 1 volume (v/w) of 2x lysis buffer was added, and the cells were thoroughly suspended. After further addition of 3 volumes (3 ml per 1g of pellet) of 1x lysis buffer the cells were broken by sonication. Lysate was then transferred to a 50ml Beckman polypropylene centrifuge tube. Cell debris and DNA from the lysate was precipitated by adding Polyethylenimine (PEI) to the tube from a 5% stock solution to a final concentration of 0.15% and incubated on ice for 15 – 30 mins for precipitation. Cell lysates were clarified by centrifugation using a JA25.50 rotor at 21,500rpm for 60 min at 4°C. The cleared supernatant was collected and filtered using a 0.45um acrodisc syringe filter and transferred to a fresh 50ml-conical polypropylene tube. **Ni^{2+}-IDA IMAC (column 1):** A 25ml Econo-Pac polypropylene column (BioRad) was packed with 5ml of Ni-Sepharose resin and equilibrated in 1x lysis buffer. The cell lysate was loaded onto the column and passed through resin at gravity flow. The unbound flow-through was collected, a wash step of 10 column volumes of wash buffer was included and collected as the wash fraction. Protein was eluted in four intermediate fractions with a step gradient of 5ml imidazole elution buffers; 100mM, 150mM, 250mM and 500mM imidazole, respectively, each gradient eluted fraction was collected. IMAC fractions were analysed by SDS-PAGE gel for expected

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	molecular weight and purity, the selected eluted fractions were pooled together. **Mass spectrometry characterisation:** Identity and biotinylation efficiency was verified by mass spectrometry, using a Quadrupole-Time of flight (Q-TOF) analyser, Biotin quantitation is measured by: MW of biotinylated protein = MW of sequenced Protein + 226 Da (biotinylation modification).
Step 2: Removal of His_6 tag and Ni^{2+}-IDA reverse binding	**Enzymatic removal of histidine tag:** Selected, pooled eluted fractions from Ni^{2+}-IDA IMAC were concentrated using a 50,000 Amicon-Ultra MWCO centrifugal concentrator to approximately 10mls in volume. Cleavage of the histidine tag was performed by the addition of His_8-taggedTEV protease in a 1:20 w/w ratio to the pooled eluted protein fractions. The protein sample plus TEV was transferred to Snakeskin dialysis tubing (3,500 MWCO) and dialysed in 1L Gel Filtration buffer overnight at 4°C on a magnetic stirrer plate for digestion. **Ni^{2+}-IDA Rebinding column (Column 2):** To remove uncleaved protein and other contaminants, the dialysate was passed through a second IMAC column. 1ml of packed Ni-Sepharose resin was added to a 25ml Econo-Pac polypropylene column (BioRad), washed in 3 CV of distilled deionized water then equilibrated in 5 column volumes of gel filtration buffer. The cleaved, dialyzed protein sample was loaded and passed through the column by gravity flow. The flow through was collected and combined with two 2 CV washes in GF buffer gel filtration buffer.(do you not do an intermediate wash?) for completeness, the column was finally eluted with a 2 CV wash of 500mM imidazole elution buffer. All fractions were analysed for verification of cleaved protein and purity by SDS-PAGE gel and mass spectrometry characterisation.
Step 3: SEC: Gel filtration	**Size Exclusion Chromatography (SEC) (Column 3):** Verified protein from rebinding column 2 was concentrated to less than 5 ml, using an Amicon-Ultra 10,000 MWCO centrifugal concentrator. The GF column (Superdex S200 16/60 HiLoad, 120ml on an ÄKTA Express chromatography system) was equilibrated in gel filtration buffer. The concentrated protein sample was loaded onto the GF column for further purification; protein was fractionated on the column in GF buffer at 1ml/min and 0.5ml fractions were collected at the A280 peaks. Peak fractions were analysed by SDS-PAGE gel, some impurities and contaminating bands were observed on the gel analysis. Peak GF fractions containing least impurities were collected, pooled and concentrated for an additional second GF purification step, attempting to reduce contaminants and increase purity of protein to >90%. Protein purity was improved after additional GF step with protein purity increasing to >90%. Selected peak GF fractions containing high protein concentration and purity were collected and pooled for the protein final sample.
Step 4: Protein storage	**Protein concentration mg/ml and storage:** Protein was concentrated using a 50,000 Amicon-Ultra MWCO centrifugal concentrator and final protein concentration was determined from the absorbance at 280nm,

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	measured using a nanodrop spectrometer (Nanodrop ND-1000, NanoDrop Technologies). Final protein concentration was measured at 1mg/ml with a final volume of 3mls. Protein was transferred into 200 µl aliquots, flash-frozen in liquid nitrogen and stored at -80°C. The final storage buffer was 20mM Hepes, pH 7.5, 500mM NaCl, 5% v/v glycerol, 1 mM TCEP

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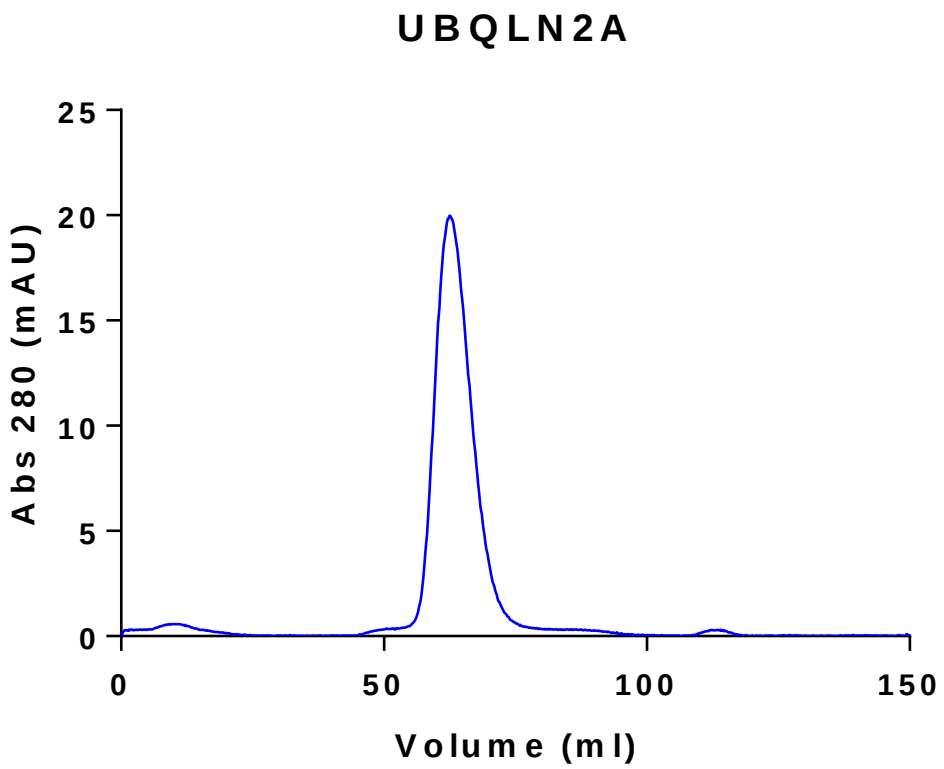
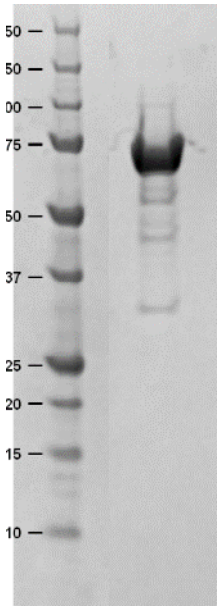
Gel Filtration Chromatogram (Superdex S200)



UBQLN2A-c000, Maximum peak elution time was 62.565ml with absorbance at 280nm of 19.978 mAU. SEC analysis validates protein is a dimer.

Final Sample SDS-PAGE



Protein	Expected mass + Biotinylation (Da)	Final concentration (mg/ml)
UBQLN2A-c000	68400	1

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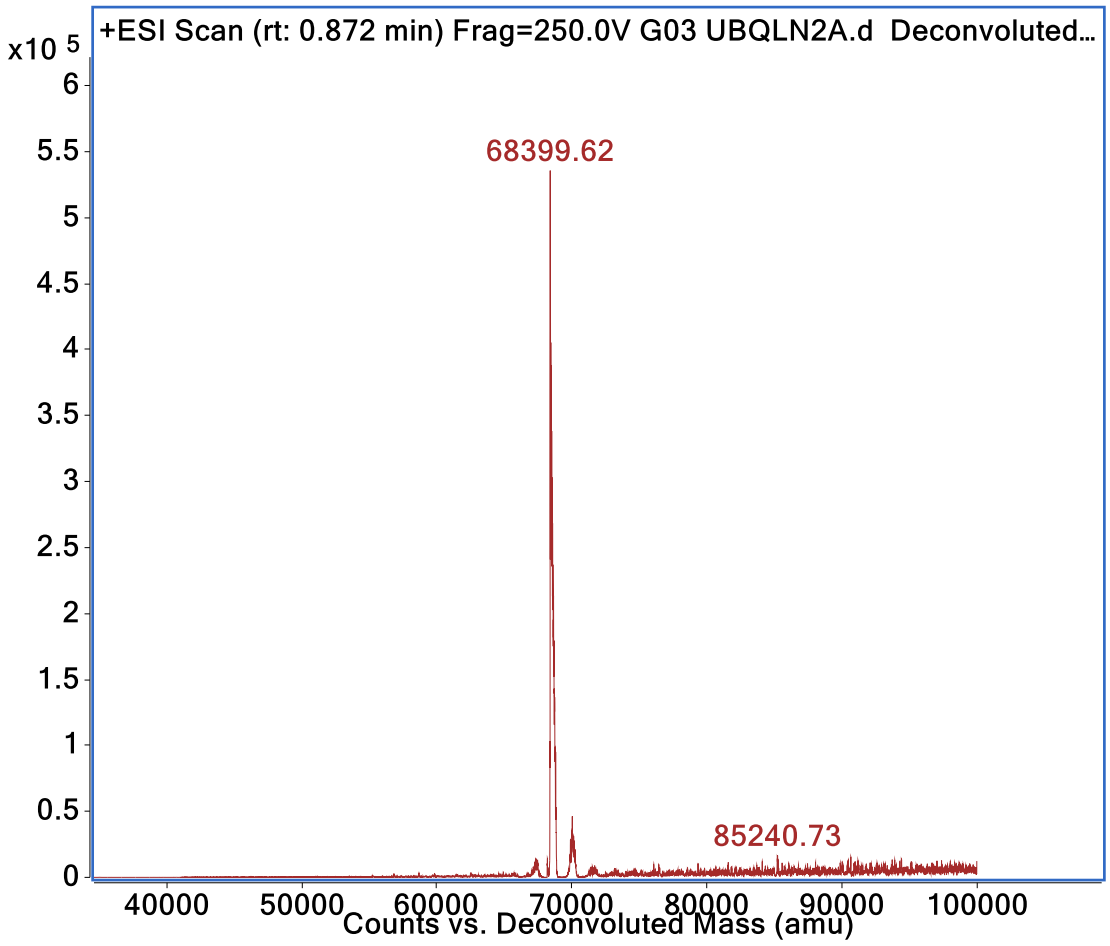
Mass Spectrometry characterisation

Protein	Vector	Expected mass cleaved + Biotinylation (Da)	Observed mass	Comments
UBQLN2A-c000	pNIC-Bio3	68400	68400	100% Biotinylated



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