Entry Clone Source: MGC

Entry Clone Accession: IMAGE:4791972

SGC Construct ID: KLHL2A-c009

GenBank GI number: gi|21359896

Vector: pNIC28-Bsa4. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

TACTTCCAATCCATGAGTGTCCGGACCCGG
CTGAGGACACCCATGAACCTTCCCAAATTG
ATGGTGGTGGTTGGGGGCCAAGCACCAAAG
GCTATCCGGAGTGTGGAATGCTATGACTTT
AAAGAAGAAAGGTGGCACCAAGTAGCAGAG
TTGCCTTCCAGGAGGTGCAGGGCAGGCATG
GTCTACATGGCTGGACTTGTTTTTGCTGTT
GGTGGCTTTAATGGCTCATTAAGAGTTCGC
ACTGTAGATTCCTACGACCCTGTGAAGGAC
CAGTGGACCAGCGTTGCTAACATGAGAGAC
CGGAGAAGCACTTTGGGAGCTGCTGTGTTA
AATGGATTATTATACGCTGTGGGAGGCTTT
GATGGGAGTACAGGTTTGTCATCTGTGGAA
GCATACAACATAAAGTCTAATGAGTGGTTT
CATGTAGCTCCCATGAATACAAGGAGGAGC
AGTGTTGGTGTGGGTGTTGTTGGAGGTTTG
CTCTATGCTGTAGGAGGTTATGATGTAGCA
TCACGTCAGTGTCTTAGCACAGTAGAATGC
TATAATGCTACAACAAATGAGTGGACCTAT
ATAGCAGAAATGAGCACCAGGCGGAGTGGA
GCAGGTGTTGGTGTGTTAAACAATTTATTG
TATGCTGTAGGAGGTCATGATGGCCCTTTA
GTACGAAAAAGTGTTGAAGTATATGATCCC
ACCACTAACGCATGGAGACAGGTTGCAGAT
ATGAACATGTGCAGAAGAAATGCAGGAGTT
TGTGCAGTTAATGGTCTGTTATATGTTGTT
GGAGGGGATGATGGTTCCTGTAACTTGGCG
TCAGTAGAATATTATAACCCAACAACCGAT
AAATGGACAGTTGTGTCATCGTGTATGAGC
ACAGGGAGAAGTTATGCAGGGGTCACAGTT
ATTGATAAACGATTATGACAGTAAAGGTGG
ATA

Final protein sequence (tag sequence in lower case)

mhhhhhhssgvdlgtenlyfqSMSVRTRLR
TPMNLPKLMVVVGGQAPKAIRSVECYDFKE
ERWHQVAELPSRRCRAGMVYMAGLVFAVGG
FNGSLRVRTVDSYDPVKDQWTSVANMRDRR
STLGAAVLNGLLYAVGGFDGSTGLSSVEAY
NIKSNEWFHVAPMNTRRSSVGVGVVGGLLY
AVGGYDVASRQCLSTVECYNATTNEWTYIA
EMSTRRSGAGVGVLNNLLYAVGGHDGPLVR
KSVEVYDPTTNAWRQVADMNMCRRNAGVCA

VNGLLYVVGGDDGSCNLASVEYYNPTTDKW
TVVSSCMSTGRSYAGVTVIDKRL
Note the MGC clone has a sequence change (Val457) compared to the Uniprot entry O95198 (Gly457).

Host: BL21(DE3)-R3-pRARE2

Expression protocol:
Glycerol stock was used to inoculate 120 ml of TB medium (supplemented with 50 µg/ml kanamycin).This starter culture was grown overnight at 37°C and used to inoculate 7 x 1 liter TB culture. The cells were cultured at 37°C with vigorous shaking (160 rpm) until the culture reached an OD_{600} of 1.5. At that point temperature was reduced to 18°C, and cells were induced with IPTG at a concentration of 0.2 mM, and cultured o/n. Cells were harvested at 9000 x g for 10 minutes and the cell pellet of 7L was resuspended in 250 ml of lysis buffer and stored at -80°C until further use.
Lysis buffer : 500 mM NaCl, 5% glycerol, 50 mM HEPES pH 7.5, 20 mM imidazole, Complete EDTA-free protease inhibitor (Roche, 1tablet / 50ml).

Extraction method: Cell pellets from 7 liter were thawed and re-suspended in Lysis buffer, and lysed in a high pressure homogeniser and then centrifuged at 4°C for 60 minutes at 48 000 x g. The supernatant was further clarified by filtration (0.45 mm) before loaded on the 5 ml HisTrap FF, column (GE heathcare).

Column 1: DE52, Whatmann
Anion-exchange for Nucleic acid removal with DEAE cellulose (DE52, Whatmann)10 g of resin was suspended in 50 ml 1 M NaCl, and then applied onto a 2.5 x 20 cm column. The resin was then equilibrated with 50 ml binding buffer prior to loading the sample.

Column 1 Buffers:
Binding buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole, 0.1mM TCEP
Wash buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 25 mM imidazole, 0.1mM TCEP

Column 1 Procedure: The supernatant was first applied onto the column by gravity flow, which was followed by a wash with 50 ml wash buffer. The column flow-through was collected for loading on column 2 (Ni-affinity).

Column 2: HisTrap FF, 5 ml (GE healthcare)

Column 2 Buffers: Lysis buffer**:** 500 mM NaCl, 5% glycerol, 50 mM HEPES pH 7.5, 20 mM imidazole, 0.5 mM TECP; Wash buffer: 500 mM NaCl, 5% glycerol, 50 mM HEPES pH 7.5, 40 mM imidazole, 0.5 mM TECP; Elution buffer: 500 mM NaCl, 5% glycerol, 50 mM HEPES pH 7.5, 250 mM imidazole, 0.5 mM TECP

Column 2 Procedure: The centrifuged cell extract was loaded on the column at 5 ml/min on an ÄKTA-Xpress system (Affinity/Gel Filtration, GE heathcare). The column was then washed with 10 column volumes of lysis buffer, 10 column volumes of wash buffer, and then eluted with 3 column volume of elution buffer at 5 ml/min. . The eluted peak of A280 was automatically collected into capillary loops and and loaded on the Hiload 16/60 Superdex 200.

Column 3: Size Exclusion Chromatography (SEC) Hiload 16/60 Superdex 200 prep grade 120 ml (GE/Amersham Biosciences)

Column 3 Buffers: Gelfiltration bufffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 0.5mM TCEP
Column 3 Procedure: AKTA Xpress Affinity/Gel Filtration. The eluted fractions from the Ni-affinity Histrap columns were loaded on the gel filtration column in GF buffer at 1ml/min. Eluted protein was collected in 1.8 ml fractions in a 96 well bloc and analyzed by SDS-PAGE.
TEV cleavage: The gel filtration fractions containing KLHL2 were pooled and TEV protease (6 mg/ml) was added. The digestion was left overnight at 4°C and cleavage was examined by SDS page, before passing the reaction mixture through Ni-NTA resin.
Column 4: 250 ml Ni-resin drip column
Column 4 Procedure: The combined KLHL2A (TEV cleaved) samples (identified by SDS PAGE) were passed through a drip column containing 250 ul Ni-sepharose resin to remove the TEV protease which contained a non-cleavable hexahistidine tag.
Concentration: The flow through fraction from the Ni-resin was collected and concentrated using Amicon Ultra 10 kDa cut off, Millipore concentrator to 9.9 mg/ml. The protein was frozen in liquid nitrogen and stored at -80°C.
Mass spectrometry characterization: The expected mass for the cleaved KLHL2 protein was 32795.4 kDa. LC-ESI-MS TOF analysis of the TEV-cleaved KLHL2 protein showed three peaks of 31709.1 kDa, 31978.kDa and 32235.8 kDa corresponding exactly to the loss of 9, 7 and 5 residues from the protein N-terminus.
Crystallisation: Crystals were grown from sitting drops comprising 50nl KLHL2 and 100nl of reservoir solution (0.2M ammonium sulphate, 0.1M MES pH 6.5, 30% PEG 5000 MME, 0.18 M sodium thiocyanate) at 20°C. Crystals were transferred briefly to reservoir solution supplemented with 25% (v/v) ethylene glycol prior to vitrification in liquid nitrogen.
Data collection: Data Collection Resolution: 2.0Å; X-ray Source: Diamond Light Source, station I24, using monochromatic radiation at wavelength 0.979 Å