Entry Clone Source: Stefan

Entry Clone Accession: n/a

SGC Construct ID: HSP90B1A-c028

GenBank GI number: gi|20149594

Vector: pNIC-CTHF. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

TTAAGAAGGAGATATACTATGAAGAC
CAAGCCTATTTGGACCAGAAACCCTG
ATGACATCACCCAAGAGGAGTATGGA
GAATTCTACAAGAGCCTCACTAATGA
CTGGGAAGACCACTTGGCAGTCAAGC
ACTTTTCTGTAGAAGGTCAGTTGGAA
TTCAGGGCATTGCTATTTATTCCTCG
TCGGGCTCCCTTTGACCTTTTTGAGA
ACAAGAAGAAAAAGAACAACATCAAA
CTCTATGTCCGCCGTGTGTTCATCAT
GGACAGCTGTGATGAGTTGATACCAG
AGTATCTCAATTTTATCCGTGGTGTG
GTTGACTCTGAGGATCTGCCCCTGAA
CATCTCCCGAGAAATGCTCCAGCAGA
GCAAAATCTTGAAAGTCATTCGCAAA
AACATTGTTAAGAAGTGCCTTGAGCT
CTTCTCTGAGCTGGCAGAAGACAAGG
AGAATTACAAGAAATTCTATGAGGCA
TTCTCTAAAAATCTCAAGCTTGGAAT
CCACGAAGACTCCACTAACCGCCGCC
GCCTGTCTGAGCTGCTGCGCTATCAT
ACCTCCCAGTCTGGAGATGAGATGAC
ATCTCTGTCAGAGTATGTTTCTCGCA
TGAAGGAGACACAGAAGTCCATCTAT
TACATCACTGGTGAGAGCAAAGAGCA
GGTGGCCAACTCAGCTTTTGTGGAGC
GAGTGCGGAAACGGGGCTTCGAGGTG
GTATATATGACCGAGCCCATTGACGA
GTACTGTGTGCAGCAGCTCAAGGAAT
TTGATGGGAAGAGCCTGGTCTCAGTT
ACCAAGGAGGGTCTGGAGCTGGCAGA
GAACCTCTACTTCCAATC

Final protein sequence (Tag sequence in lowercase):

MKTKPIWTRNPDDITQEEYGEFYKSL
TNDWEDHLAVKHFSVEGQLEFRALLF
IPRRAPFDLFENKKKKNNIKLYVRRV
FIMDSCDELIPEYLNFIRGVVDSEDL
PLNISREMLQQSKILKVIRKNIVKKC
LELFSELAEDKENYKKFYEAFSKNLK
LGIHEDSTNRRRLSELLRYHTSQSGD
EMTSLSEYVSRMKETQKSIYYITGES
KEQVANSAFVERVRKRGFEVVYMTEP

IDEYCVQQLKEFDGKSLVSVTKEGLE
Laenlyfq^shhhhhhdykddddk

^ TEV cleave site

Tags and additions: TEV Cleavable C-terminal hexahistidine tag.

Host: BL21 (DE3)R3-pRARE2 (Phage resistant strain).

Growth medium, induction protocol: 10µl of BL21(BE3)-R3-pRARE2 glycerol stock were inoculated into 5ml of TB with 50µg/ml of kanamycin and 34µg/ml chloramphenicol and grown overnight at 37°C, 200rpm. 10ml of overnight culture were added to 1L of TB with 50µg/ml kanamycin and incubated at 37°C, 160rpm. After the OD_{600} reached 1.0, the temperature was dropped to 18°C and 500µl of 1M IPTG was added to the final concentration of ~0.5 mM. The culture was then incubated with shaking overnight at 18°C, 160 rpm. The following morning the 6L culture was harvested and centrifuged for 10min at 40000rpm. Supernatant was discareded and cell pellets were resuspended in 80ml of a lysis buffer and frozen at -80°C.
Lysis buffer: 50 mM HEPES pH 7.5; 500 mM NaCl; 5 mM Imidazole; 5% Glycerol; 1 mM PMSF.
Extraction buffer, extraction method: The thawed cells were broken by 5 passes at 16000 psi through a high pressure homogeniser followed by centrifugation for 45 min at 15000rpm. The supernatant was kept for further purification.

Column 1: Ni-sepharose.

Column 1 Buffer:
Binding Buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 20 mM Imidazole; 5% glycerol; 1 mM PMSF; 0.5 mM TCEP.
Washing Buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 40 mM Imidazole; 5% glycerol; 1 mM PMSF; 0.5 mM TCEP.
Elution Buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 250 mM Imidazole; 5% glycerol; 0.5 mM TCEP.

Column 1 Procedure: 6ml of 50% Ni-sepharose slurry (Amersham) was applied onto a 1.5 x 10cm column. The column was first washed with deionised distilled H_2O, and then equilibrated with 15ml binding buffer. The supernatant was loaded onto the column and the flow through was collected. The column was washed with 30ml of binding buffer and then protein was eluted with 15ml of washing buffer and 10ml of elution buffer.

Enzymatic treatment: The His-tag was cleaved with 1mg TEV per 40mg target protein at 4°C overnight. The cleaved protein was passed again through Ni-sepharose using buffers as above (initial sample diluted 10-fold to decrease imidazole concentration). The flow-through was collected, concentrated to 5ml and loaded onto the GF column.

Column 2: Superdex 75 HiLoad 16/60 (Amersham).

Column 2 Buffers: 10 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 0.5 mM TCEP.

Column 2 Procedure: Gel filtration was carried out on an ÄKTA Purifier. The extinction at 280nm was monitored and fractions were collected and analysed by SDS-PAGE. Pulled fractions were characterised by mass spectrometry.

Protein concentration: Using MilliPore concentrators with 10kDa cutoff, the sample was concentrated to 11.6mg/ml. concentrations were determined from the absorbance at 280nm using NanoDrop.

Mass spectrometry characterization: The purified native protein was homogeneous and had an experimental mass of 31785Da matching the expected MW = 31784.4Da.Masses were determined by LC-MS, using an Agilent LC/MSD TOF system with reversed-phase HPLC coupled to electrospray ionisation and an orthogonal time-of-flight mass analyser. Proteins were desalted prior to mass spectrometry by rapid elution off a C3 column with a gradient of 5-95% isopropanol in water with 0.1% formic acid.

Crystallisation: Microseeds were prpared from initial crystals obtained from a condition containing 25.5% PEG3350, 0.17M $(NH_4)_2SO_4$, 15% glycerol. Diffraction-quality crystals were then grown at 20°C in 170µl sitting drops containing 75µl protein (11.6mg/ml), 20µl seed and 75µl of reservoir solution containing 25.5% PEG3350, 0.17M $(NH_4)_2SO_4$, 15% glycerol. Crystals were vitrified in well solution supplemented with 20% ethylene glycol.

Data Collection: The native crystal diffracted to a resolution of 2.28 Å.
X-ray source: Diamond Light Source beamline I04. .