Entry Clone Source: MGC

Entry Clone Accession: IMAGE:40125802

SGC Construct ID: TP73A-c009

GenBank GI number: gi|4885645

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

Amplified construct sequence:

AGGGGACGCAGCGAAACCGGGGCCCGCGCC
AGGCCAGCCGGGACGGACGCCGATGCCCGG
GGCTGCGACGGCTGCAGAGCGAGCTGCCCT
CGGAGGCCGGCGTGGGGAAGATGGCCCAGT
CCACCGCCACCTCCCCTGATGGGGGCACCA
CGTTTGAGCACCTCTGGAGCTCTCTGGAAC
CAGACAGCACCTACTTCGACCTTCCCCAGT
CAAGCCGGGGGAATAATGAGGTGGTGGGCG
GAACGGATTCCAGCATGGACGTCTTCCACC
TGGAGGGCATGACTACATCTGTCATGGCCC
AGTTCAATCTGCTGAGCAGCACCATGGACC
AGATGAGCAGCCGCGCGGCCTCGGCCAGCC
CCTACACCCCAGAGCACGCCGCCAGCGTGC
CCACCCACTCGCCCTACGCACAACCCAGCT
CCACCTTCGACACCATGTCGCCGGCGCCTG
TCATCCCCTCCAACACCGACTACCCCGGAC
CCCACCACTTTGAGGTCACTTTCCAGCAGT
CCAGCACGGCCAAGTCAGCCACCTGGACGT
ACTCCCCGCTCTTGAAGAAACTCTACTGCC
AGATCGCCAAGACATGCCCCATCCAGATCA
AGGTGTCCACCCCGCCACCCCCAGGCACTG
CCATCCGGGCCATGCCTGTTTACAAGAAAG
CGGAGCACGTGACCGACGTCGTGAAACGCT
GCCCCAACCACGAGCTCGGGAGGGACTTCA
ACGAAGGACAGTCTGCTCCAGCCAGCCACC
TCATCCGCGTGGAAGGCAATAATCTCTCGC
AGTATGTGGATGACCCTGTCACCGGCAGGC
AGAGCGTCGTGGTGCCCTATGAGCCACCAC
AGGTGGGGACGGAATTCACCACCATCCTGT
ACAACTTCATGTGTAACAGCAGCTGTGTAG
GGGGCATGAACCGGCGGCCCATCCTCATCA
TCATCACCCTGGAGATGCGGGATGGGCAGG
TGCTGGGCCGCCGGTCCTTTGAGGGCCGCA
TCTGCGCCTGTCCTGGCCGCGACCGAAAAG
CTGATGAGGACCACTACCGGGAGCAGCAGG
CCCTGAACGAGAGCTCCGCCAAGAACGGGG
CCGCCAGCAAGCGTGCCTTCAAGCAGAGCC
CCCCTGCCGTCCCCGCCCTTGGTGCCGGTG
TGAAGAAGCGGCGGCATGGAGACGAGGACA
CGTACTACCTTCAGGTGCGAGGCCGGGAGA
ACTTTGAGATCCTGATGAAGCTGAAAGAGA
GCCTGGAGCTGATGGAGTTGGTGCCGCAGC
CACTGGTGGACTCCTATCGGCAGCAGCAGC

AGCTCCTACAGAGGCCGAGTCACCTACAGC
CCCCGTCCTACGGGCCGGTCCTCTCGCCCA
TGAACAAGGTGCACGGGGGCATGAACAAGC
TGCCCTCCGTCAACCAGCTGGTGGGCCAGC
CTCCCCCGCACAGTTCGGCAGCTACACCCA
ACCTGGGGCCCGTGGGCCCCGGGATGCTCA
ACAACCATGGCCACGCAGTGCCAGCCAACG
GCGAGATGAGCAGCAGCCACAGCGCCCAGT
CCATGGTCTCGGGGTCCCACTGCACTCCGC
CACCCCCCTACCACGCCGACCCCAGCCTCG
TCAGTTTTTTAACAGGATTGGGGTGTCCAA
ACTGCATCGAGTATTTCACCTCCCAAGGGT
TACAGAGCATTTACCACCTGCAGAACCTGA
CCATTGAGGACCTGGGGGCCCTGAAGATCC
CCGAGCAGTACCGCATGACCATCTGGCGGG
GCCTGCAGGACCTGAAGCAGGGCCACGACT
ACAGCACCGCGCAGCAGCTGCTCCGCTCTA
GCAACGCGGCCACCATCTCCATCGGCGGCT
CAGGGGAACTGCAGCGCCAGCGGGTCATGG
AGGCCGTGCACTTCCGCGTGCGCCACACCA
TCACCATCCCCAACCGCGGCGGCCCAGGCG
GCGGCCCTGACGAGTGGGCGGACTTCGGCT
TCGACCTGCCCGACTGCAAGGCCCGCAAGC
AGCCCATCAAGGAGGAGTTCACGGAGGCCG
AGATCCACTGAGGGCCTCGCCTGGCTGCAG
CCTGCGCCACCGCCCAGAGACCCAAGCTGC
CTCCCCTCTCCTT

Final protein sequence

MAPVIPSNTDYPGPHHFEVTFQQSSTAKSA
TWTYSPLLKKLYCQIAKTCPIQIKVSTPPP
PGTAIRAMPVYKKAEHVTDVVKRCPNHELG
RDFNEGQSAPASHLIRVEGNNLSQYVDDPV
TGRQSVVVPYEPPQVGTEFTTILYNFMCNS
SCVGGMNRRPILIIITLEMRDGQVLGRRSF
EGRICACPGRDRKADEDHYREAENLYFQSH
HHHHHDYKDDDDK

Tags and additions: ENLYFQ*SHHHHHHDYKDDDDK, TEV-cleavable (*) C-terminal hexahistidine and FLAG tag.

Host: BL21(DE3)-R3-pRARE2

Growth medium, induction protocol: A glycerol stock was used to inoculate a 10ml starter culture containing LB media with 50µg/ml Kanamycin and 34 µg/ml chloramphenicol. The starter culture was grown overnight at 37°C with shaking at 200 rpm. The following morning, two flasks containing 1 L LB/kanamycin/chloramphenicol were each inoculated with 5 ml of the starter culture. Cultures were incubated at 37°C with shaking at 180 rpm until an OD_{600}nm ≥ 0.6 was reached. The flasks were then cooled down to 18°C. Protein expression was induced by addition of IPTG to a final concentration of 0.2mM and expression carried out overnight. Cells were harvested by centrifugation at 6000 rpm at 4°C for 15 min. Cell pellets from each flask were resuspended in 30ml binding buffer (50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole), transferred to 50 ml tubes, and stored at -20°C.

Extraction buffer, extraction method: The frozen cells were thawed and 0.5mM TCEP, 1mM PMSF added to the cell suspension. The cells were lysed by ultrasonication over 12 min with the sonicator pulsing ON for 4 sec and OFF for 8 sec. The cell lysate was spun down by centrifugation at 20000 rpm at 4°C for 1 h. The supernatant was recovered for purification.

Column 1: Anion-exchange for Nucleic acid removal with DEAE cellulose (DE52, Whatmann)
10 g of resin was suspended in 100 ml 2.5 M NaCl, and then applied onto a 2.5 x 20 cm column. The resin was then equilibrated with 100 ml binding buffer prior to loading the sample.

Column 1 Buffers: Binding buffer: 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole **Wash buffer:** 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 30mM Imidazole

Column 1 Procedure: The supernatant was first applied onto the column by gravity flow, which was followed by a wash with 100 ml wash buffer. The column flow-through and wash was directly applied onto the following Ni-sepharose column.

Column 2: Ni-Affinity Chromatography
5ml of 50 % Ni-sepharose slurry (Amersham) was applied onto a 1.5 x 10 cm column. The column was first washed with deionised distilled H_2O, and then equilibrated with binding buffer.

Column 2 Buffers:
Binding buffer: 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole
Wash buffer: 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 30mM Imidazole
Elution buffer: 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 50 to 250mM Imidazole

Column 2 Procedure: The supernatant was applied by gravity flow onto the Ni-sepharose column. The column was washed with wash buffer to remove nonspecifically binding proteins. The bound target protein was eluted by applying a step gradient of imidazole (5 ml fractions of elution buffer supplemented with 50mM, 100mM, 150mM and 250mM imidazole). The protein content of collected fractions was visualized using SDS-PAGE and fractions containing sufficient TP73A (relative to contaminating proteins) were pooled. 10mM DTT was added to the pooled fractions for overnight storage at 4°C.

Enzymatic treatment: TEV protease cleavage. Pooled fractions were treated with TEV protease overnight at 4°C.

Column 3: Size Exclusion Chromatography - S200 HiLoad 16/60 Superdex run on ÄKTA-Express.

Column 3 Buffers: Gel Filtration buffer: 300mM NaCl, 50mM HEPES pH 7.5, 0.5mM TCEP

Column 3 Procedure: Prior to applying the protein, the S200 16/60 column was washed and equilibrated with gel filtration buffer. Eluted protein from the Ni-sepharose column was cleaved with TEV protease and concentrated to 1ml using an Amicon Ultra-15 filter with a 10kDa cut-off. The concentrated protein was directly applied onto the equilibrated S200 16/60 column, and run at a flow-rate of 1 ml/min. Fractions were visualized using SDS-PAGE and those containing TP73A were pooled. After concentration, the protein was divided into 100µl aliquots and stored at -80°C.

Protein concentration: The protein was concentrated to a final concentration of 19mg/ml (measured by OD_{280} based on extinction coefficient 18910) in an Amicon Ultra-15 filter with

a 10 kDa cut-off.

Mass spectrometry characterization: The purified native protein was homogeneous and had an experimental mass after tag cleavage of 23184.05 consistent with loss of the N-terminal initiator methionine (calculated MW of this species = 23313.6). 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.

Crystallisation: Protein at 19mg/ml was buffered in 50mM HEPES, pH 7.5, 300mM NaCl, 0.5 mM TCEP. Crystals were grown at 20°C in 150 nl sitting drops mixing 50 nl protein solution with 100 nl of a reservoir solution containing 1.2M sodium potassium tartrate, 0.25% PEG MME 5000 and 0.1M Tris pH 9. On mounting crystals were cryo-protected with an additional 20% glycerol.

Data collection: Resolution: 1.8 Å; **X-ray source:** Diamond I02
Crystals of TP73A diffracted to a resolution of 1.8 Å (scaled resolution). A full dataset was collected at 100 K on Diamond Light Source beamline I02. Crystals belonged to the cubic space group $P4_332$ with unit-cell parameters $a=b=c=$ 110.32 Å. Only one molecule was present in the asymmetric unit. Data were indexed and integrated using iMOSFLM and scaled using SCALA. Phases were found using molecular replacement in PHASER. CHAINSAW was used to optimize the PDB entry 20CJ for use as a search model. The structure was refined and modified using alternate rounds of REFMAC5 and COOT, and the final model validated using the JCSG Quality Control Server.