Entry Clone Source: MGC

Entry Clone Accession: BC000653

SGC Construct ID: DNPEPA-c102

GenBank GI number: gi|6912248

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

Coding DNA sequence:

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CTTAAGAAGGAGATATACTATGAGCGGACA
CAGCCCCACGCGCGGGGCCATGCAGGTGGC
CATGAACGGTAAGGCCCGCAAAGAGGCGGT
GCAGACTGCGGCTAAGGAACTCCTCAAGTT
CGTGAACCGGAGTCCCTCTCCTTTCCATGC
TGTGGCTGAATGCCGCAACCGCCTTCTCCA
GGCTGGCTTCAGTGAACTCAAGGAGACTGA
GAAATGGAATATTAAGCCCGAGAGCAAGTA
CTTCATGACCAGGAACTCCTCCACCATCAT
AGCTTTTGCTGTAGGGGGCCAGTACGTTCC
TGGCAATGGCTTCAGCCTCATCGGGGCCCA
CACGGACAGCCCCTGCCTCCGGGTGAAACG
TCGGTCTCGCCGCAGCCAGGTGGGCTTCCA
GCAAGTCGGTGTGGAGACCTATGGTGGTGG
GATCTGGAGCACCTGGTTTGACCGTGACCT
GACTCTGGCTGGACGCGTCATTGTCAAGTG
CCCTACCTCAGGTCGGCTGGAGCAGCAGCT
GGTGCACGTGGAGCGGCCCATTCTTCGCAT
CCCACACCTGGCCATCCATCTGCAGCGAAA
TATCAACGAGAACTTTGGGCCCAACACAGA
GATGCATCTAGTCCCCATTCTTGCCACAGC
CATCCAGGAGGAGCTGGAGAAGGGGACTCC
TGAGCCAGGGCCTCTCAATGCTGTGGATGA
GCGGCACCATTCGGTCCTCATGTCCCTGCT
CTGTGCCCATCTGGGGCTGAGCCCCAAGGA
CATAGTGGAGATGGAGCTCTGCCTTGCAGA
CACCCAGCCTGCGGTCTTGGGTGGTGCCTA
TGATGAGTTCATCTTTGCTCCTCGGCTGGA
CAATCTGCACAGCTGCTTCTGTGCCCTGCA
GGCCTTGATAGATTCCTGTGCAGGCCCTGG
CTCCCTGGCCACAGAGCCTCACGTGCGCAT
GGTCACACTCTATGACAACGAAGAGGTGGG
GTCTGAGAGTGCACAGGGAGCACAGTCACT
GCTGACAGAGCTGGTGCTGCGGCGGATCTC
AGCCTCGTGCCAGCACCCGACAGCCTTCGA
GGAAGCCATACCCAAGTCCTTCATGATCAG
CGCAGACATGGCCCATGCTGTGCATCCCAA
CTACCTGGACAAGCATGAGGAGAACCACCG
GCCTTTATTCCACAAGGGCCCCGTGATCAA
GGTGAACAGCAAGCAACGCTATGCTTCAAA
CGCGGTGTCAGAGGCCCTGATCCGAGAGGT
GGCCAACAAAGTCAAGGTCCCCCTGCAGGA
TCTCATGGTCCGGAATGACACCCCCTGTGG
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AACCACCATTGGACCTATCTTGGCTTCTCG
GCTGGGGCTGCGGGTGCTGGATTTAGGCAG
CCCCCAACTGGCCATGCACTCTATCCGGGA
GATGGCCTGCACCACAGGAGTCCTCCAGAC
CCTCACCCTCTTCAAGGGCTTCTTTGAGCT
GTTCCCTTCTCTAGCAGAGAACCTCTACTT
CCAATCGCACCATCATCACCACCATGATTA
CAAGGATGACGACGATAAGTGAGGATCC

Tags and additions: N-terminal, TEV cleavable hexahistidine tag.

Host:*E. coli* BL21(DE3)-R3-pRARE2

Tag sequence: AENLYFQ(*)SHHHHHHDYKDDDDK

Expressed protein sequence:
MSGHSPTRGAMQVAMNGKARKEAVQTAAKE
LLKFVNRSPSPFHAVAECRNRLLQAGFSEL
KETEKWNIKPESKYFMTRNSSTIIAFAVGG
QYVPGNGFSLIGAHTDSPCLRVKRRSRRSQ
VGFQQVGVETYGGGIWSTWFDRDLTLAGRV
IVKCPTSGRLEQQLVHVERPILRIPHLAIH
LQRNINENFGPNTEMHLVPILATAIQEELE
KGTPEPGPLNAVDERHHSVLMSLLCAHLGL
SPKDIVEMELCLADTQPAVLGGAYDEFIFA
PRLDNLHSCFCALQALIDSCAGPGSLATEP
HVRMVTLYDNEEVGSESAQGAQSLLTELVL
RRISASCQHPTAFEEAIPKSFMISADMAHA
VHPNYLDKHEENHRPLFHKGPVIKVNSKQR
YASNAVSEALIREVANKVKVPLQDLMVRND
TPCGTTIGPILASRLGLRVLDLGSPQLAMH
SIREMACTTGVLQTLTLFKGFFELFPSL**AE**
NLYFQ

***** **AENLYFQ** residues originate from the vector and remain after the TEV cleavage of the hexahistidine tag.

Expression:
10ul of BL21(DE3)-R3-pRARE2 glycerol stock were inoculated into 5ml of TB with 50ug/ml kanamycin and 34ug/ml chloramphenicol and grown overnight at 37°C, 200rpm. 10ml of overnight culture were added to 1L of TB with 50ug/ml kanamycin and incubated at 37°C, 160rpm. After the OD_{600} reached 1.0, the temperature was dropped to 18°C and 500ul of 1M IPTG was added to the final concentration of ˜0.5mM. The culture was then incubated with shaking overnight at 18°C, 160rpm. The following morning the 4L cultures were harvested and centrifuged for 10min at 4000rpm. Supernatant was discarded and cell pellets were resuspended in 80ml of lysis buffer and frozen at -80°C.

Extraction: Lysis buffer: 50mM HEPES pH 7.5, 500mM NaCl, 5mM Imidazole, 5% glycerol, EDTA-free Complete (1 tablet/50ml).
The thawed cells were broken by 5 passes at 16.000 psi through a high pressure homogeniser followed by centrifugation for 45 min at 15,000rpm.

Column 1: Ni-affinity, His-Trap, 1 ml (Amersham)
Column 2: Superdex 200, HiPrep 16/60 (Amersham)

Buffers:
Start buffer: 50mM HEPES pH 7.5, 500mM NaCl, 20mM Imidazole, 5% glycerol, 1mM PMSF, 0.5mM TCEP
Washing buffer: 50mM HEPES pH 7.5, 500mM NaCl, 40mM Imidazole, 5% glycerol, 1mM PMSF, 0.5mM TCEP
Elution buffer: 50mM HEPES pH 7.5, 500mM NaCl, 5% glycerol, 250mM Imidazole, 0.5mM TCEP
GF buffer: 10mM HEPES pH 7.5, 500mM NaCl, 5% glycerol, 0.5mM TCEP

Procedure: The cell extract was loaded on the AKTA Express system. The absorbance at 280nm was monitored and fractions were collected and analyzed by SDS-PAGE. Positive fractions were pooled for the TEV cleavage.
TEV cleavage: The His-tag was cleaved with 1 mg TEV per 40 mg target protein at 4°C overnight. Uncleaved protein and His-TEV were removed by passing the sample onto IMAC Sepharose resin pre-equilibrated with GF buffer.

Column 3: Superdex 200, HiPrep 16/60 (Amersham)

Buffers:
GF buffer: 10mM HEPES pH 7.5, 500mM NaCl, 5% glycerol, 0.5mM TCEP

Procedure: TEV-cleaved protein was loaded onto the GF column. The absorbance at 280nm was monitored and fractions were collected and analyzed by SDS-PAGE. Fractions containing DNPEP were pooled and characterised by mass spectrometry.

Concentration and buffer exchange:
Using Amicon Ultra-15 concentrators with 10 kDa cutoff, the sample was concentrated to 9mg/ml. Concentrations were determined from the absorbance at 280 nm using NanoDrop.

Mass spectrometry characterization: Calculated mass of the construct was 53498. The exact mass of the protein lacking the N-terminal Met was confirmed by the mass spectrometry.

Crystallization: Crystals were grown by vapour diffusion at 20°C in 150nl sitting drops. The drops were prepared by mixing 100nl of protein solution and 50nl of precipitant consisting of 0.1 M Tris pH 8.0, 0.25 M $MgCl_2$ and 15% (w/v) of PEG 3350. Crystals were transferred to a cryo-protectant consisting of 25% glycerol and 75% well solution before flash-cooling in liquid nitrogen

Data Collection:
Resolution: 2.4Å
X-ray source: Diamond beamline IO3