Entry Clone Source: MGC

Entry Clone Accession: IMAGE:5087393

SGC Construct ID: HPDA-c103

GenBank GI number: gi|4504477

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

Amplified construct sequence:

```
CTTAAGAAGGAGATATACTATGGGGGCAAA
GCCTGAGAGAGGCCGATTCCTCCACTTCCA
CTCTGTGACCTTCTGGGTTGGCAACGCCAA
GCAGGCCGCGTCATTCTACTGCAGCAAGAT
GGGCTTTGAACCTCTAGCCTACAGGGGCCT
GGAGACCGGTTCCCGGGAGGTGGTCAGCCA
TGTAATCAAACAAGGGAAGATTGTGTTTGT
CCTCTCCTCAGCGCTCAACCCCTGGAACAA
AGAGATGGGCGATCACCTGGTGAAACACGG
TGACGGAGTGAAGGACATTGCGTTCGAGGT
GGAAGATTGTGACTACATCGTGCAGAAAGC
ACGGGAACGGGGCGCCAAAATCATGCGGGA
GCCCTGGGTAGAGCAAGACAAGTTTGGGAA
GGTGAAGTTTGCTGTGCTGCAGACGTATGG
GGACACCACACACACCCTGGTGGAGAAGAT
GAACTACATCGGCCAATTCTTGCCTGGATA
TGAGGCCCCAGCGTTCATGGACCCCCTACT
TCCTAAACTGCCCAAATGCAGTCTGGAGAT
GATCGACCACATTGTGGGAAACCAGCCTGA
TCAGGAGATGGTGTCCGCCTCCGAATGGTA
CCTGAAAAACCTGCAGTTCCACCGCTTCTG
GTCCGTGGATGACACGCAGGTGCACACGGA
ATATAGCTCTCTGCGATCCATTGTGGTGGC
CAACTATGAAGAGTCCATCAAGATGCCCAT
CAATGAGCCAGCGCCTGGCAAGAAGAAGTC
CCAGATCCAGGAATATGTGGACTATAACGG
GGGCGCTGGGGTCCAGCACATCGCTCTCAA
GACCGAAGACATCATCACAGCGATTCGCCA
CTTGAGAGAGAGAGGCCTGGAGTTCTTATC
TGTTCCCTCCACGTACTACAAACAACTGCG
GGAGAAGCTGAAGACGGCCAAGATCAAGGT
GAAGGAGAACATTGATGCCCTGGAGGAGCT
GAAAATCCTGGTGGACTACGACGAGAAAGG
CTACCTCCTGCAGATCTTCACCAAACCGGT
GCAGGACCGGCCCACGCTCTTCCTGGAAGT
CATCCAGCGCCACAACCACCAGGGTTTTGG
AGCCGGCAACTTCAACTCACTGTTCAAGGC
TTTCGAGGAGGAGCAGAACCTGCGGGGTAA
CCTCACCAACATGGAGACCAATGGGGTGGT
GCCCGGCATGGCAGAGAACCTCTACTTCCA
ATCGCACCATCATCACCACCATGATTACAA
GGATGACGACGATAAGTGAGGATCC
```

Final protein sequence (tag sequence in lowercase):

```
MGAKPERGRFLHFHSVTFWVGNAKQAASFY
CSKMGFEPLAYRGLETGSREVVSHVIKQGK
IVFVLSSALNPWNKEMGDHLVKHGDGVKDI
AFEVEDCDYIVQKARERGAKIMREPWVEQD
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KFGKVKFAVLQTYGDTTHTLVEKMNYIGQF
LPGYEAPAFMDPLLPKLPKCSLEMIDHIVG
NQPDQEMVSASEWYLKNLQFHRFWSVDDTQ
VHTEYSSLRSIVVANYEESIKMPINEPAPG
KKKSQIQEYVDYNGGAGVQHIALKTEDIIT
AIRHLRERGLEFLSVPSTYYKQLREKLKTA
KIKVKENIDALEELKILVDYDEKGYLLQIF
TKPVQDRPTLFLEVIQRHNHQGFGAGNFNS
LFKAFEEEQNLRGNLTNMETNGVVPGMaen
lyfq(*)shhhhhhdykddddk
Residues **aenlyfq** originate from the vector and remain after the TEV cleavage of the hexahistidine tag.

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

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

Expression: 10ul of BL21(DE3)-R3-pRARE2 glycerol stock were inoculated into 5ml of TB with 50ug/ml of 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 culture was harvested and centrifuged for 10min at 4000rpm. Supernatant was discarded and cell pellets were resuspended in 80ml of a lysis buffer and frozen at -80°C.

Extraction: Lysis buffer: 50mM HEPES pH 7.5, 500mM NaCl, 5mM Imidazole, 5% glycerol + 1mM PMSF. 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: Binding 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 extinction at 280nm was monitored and fractions were collected and analyzed by SDS-PAGE. Positive fractions were pooled for TEV cleavage.
TEV cleavage: The His-tag was cleaved with 1 mg TEV per 40 mg target protein at 4°C overnight. The protein was further purified on IMAC Sepharose using buffers as above.

Concentration and buffer exchange:
Using Amicon Ultra-15 concentrators with 30 kDa cutoff, the sample was buffer-exchanged into 10 mM Tris pH 8.5, 100 mM NaCl and concentrated to 14.91 mg/ml. Concentrations were determined from the absorbance at 280 nm using NanoDrop.

Mass spectrometry characterization: Calculated mass of the construct was 45105. The exact mass for the protein lacking the N-terminal methionine was confirmed by the mass spectrometry.

Crystallization: Crystals were grown by vapour diffusion in sitting drops at 4°C. A sitting drop consisting of 50 nl protein and 100 nl well solution was equilibrated against well solution containing 0.2M Na(acetate); 0.1M Bis-Tris Propane pH 6.5; 20% PEG 3350, 10% Ethylene Glycol. Crystals were cryo-protected in 10% Ethylene Glycol and 90% well solution before flash-cooling in liquid nitrogen.

Data Collection: Resolution: 1.75Å; **X-ray source:** Diamond microfocus