Entry Clone Source: MGC
Entry Clone Accession: IMAGE:4824753
SGC Construct ID: GAPDHSA-c105
GenBank GI number: gi|7657116
Vector: pNIC-CTHF. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

CTTAAGAAGGAGATATACTATGGTGT
CTGTGGCCCGGGAGCTGACTGTGGGC
ATCAATGGATTTGGACGCATCGGTCG
CCTGGTCCTGCGCGCCTGCATGGAGA
AGGGTGTTAAGGTGGTGGCTGTGAAT
GATCCATTCATTGACCCGGAATACAT
GGTGTACATGTTTAAGTATGACTCCA
CCCACGGCCGATACAAGGGAAGTGTG
GAATTCAGGAATGGACAACTGGTCGT
GGACAACCATGAGATCTCTGTCTACC
AGTGCAAAGAGCCCAAACAGATCCCC
TGGAGGGCTGTCGGGAGCCCCTACGT
GGTGGAGTCCACAGGCGTGTACCTCT
CCATACAGGCAGCTTCGGACCACATC
TCTGCAGGTGCTCAACGTGTGGTCAT
CTCCGCGCCCTCACCGGATGCACCAA
TGTTCGTCATGGGTGTCAATGAAAAT
GACTATAACCCTGGCTCCATGAACAT
TGTGAGCAACGCGTCCTGCACCACCA
ACTGTTTGGCTCCCCTCGCCAAAGTC
ATCCACGAGCGATTTGGGATCGTGGA
AGGGTTGATGACCACAGTCCATTCCT
ACACGGCCACCCAGAAGACAGTGGAC
GGGCCATCAAGGAAGGCCTGGCGAGA
TGGGCGGGGTGCCCACCAGAACATCA
TCCCAGCCTCCACTGGGGCTGCGAAA
GCTGTGACCAAAGTCATCCCAGAGCT
CAAAGGGAAGCTGACAGGGATGGCGT
TCCGGGTACCAACCCCGGATGTGTCT
GTCGTGGACCTGACCTGCCGCCTCGC
CCAGCCTGCCCCCTACTCAGCCATCA
AGGAGGCTGTAAAAGCAGCAGCCAAG
GGGCCCATGGCTGGCATCCTTGCCTA
CACCGAGGATGAGGTCGTCTCTACGG
ACTTCCTCGGTGATACCCACTCGTCC
ATCTTCGATGCTAAGGCCGGCATTGC
GCTCAATGACAATTTCGTGAAGCTCA
TTTCATGGTACGACAACGAATATGGC
TACAGTCACCGGGTGGTCGACCTCCT
CCGCTACATGTTCAGCCGAGACGCAG
AGAACCTCTACTTCCAATCGCACCAT
CATCACCACCATGATTACAAGGATGA
CGACGATAAGTGAGGATCC

Final protein sequence (Tag sequence in lowercase):

MVSVARELTVGINGFGRIGRLVLRAC
MEKGVKVVAVNDPFIDPEYMVYMFKY
DSTHGRYKGSVEFRNGQLVVDNHEIS
VYQCKEPKQIPWRAVGSPYVVESTGV
YLSIQAASDHISAGAQRVVISAPSPD
APMFVMGVNENDYNPGSMNIVSNASC
TTNCLAPLAKVIHERFGIVEGLMTTV

HSYTATQKTVDGPSRKAWRDGRGAHQ
NIIPASTGAAKAVTKVIPELKGKLTG
MAFRVPTPDVSVVDLTCRLAQPAPYS
AIKEAVKAAAKGPMAGILAYTEDEVV
STDFLGDTHSSIFDAKAGIALNDNFV
KLISWYDNEYGYSHRVVDLLRYMFSR
Daenlyfq^shhhhhhdykddddk

^ TEV cleave site

Tags and additions: C-terminal Histidine-tag with TEV protease cleavage site.

Host: BL21 (DE3) R3-pRARE2.

Growth medium, induction protocol: 10µl of a glycerol stock was inoculated into 5ml of TB medium (supplemented with 50µg/ml Kanamycin, 34µg/ml Chloramphenicol) and cultured at 37°C o/n in a shaking incubator (275 rpm). Next day 0.75 ml of o/n culture was used to inoculate 1 litre of TB medium (6 x) and grown at 37°C with vigorous shaking (160 rpm) until the culture reaches an OD_{600} of 1.5. Temperature was reduced to 18°C, and cells were induced with IPTG at a concentration of 0.5 mM, and further cultivated for 16 hrs. Cells were harvested by centrifugation at 6500 rpm for 10 min, and the cell pellet was stored at -20°C until further use.
Lysis buffer: 500 mM NaCl; 50 mM imidazole; 5 mM imidazole; 5% glycerol; Complete® protease inhibitors (Roche, 1 tbl/50ml).
Extraction buffer, extraction method: Frozen cell pellets were thawed and resuspended in a total volume of 30-40ml of lysis buffer, and disrupted by using sonicator, and a supernatant containing the target protein was obtained by centrifugation at 21,000 rpm for 45 minutes.

Column 1: Ni-Sepharose 6 Fast Flow

Column 1 Buffer:
lysis buffer: 50mM mM 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; 250mM Imidazole.

Column 1 Procedure: The column was packed with 2ml of Ni-Sepharose 6 Fast Flow slurry and equilibrated with 15ml of binding buffer. The supernatant was loaded onto the column and the column was washed with 20ml of binding buffer and then 20ml of wash buffer. The protein was eluted with 10ml of elution buffer.

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

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

Column 2 Procedure: The eluted protein from the Ni-Sepharose column was loaded on the gel filtration column in GF buffer at 1.0ml/min on an ÄKTA Purifier system. Eluted proteins were collected in 1ml fractions.

Enzymatic treatment: TEV cleaved.

Column 3: Ni-NTA (TEV clean up).

Column 3 Buffers: 10 mM HEPES pH 7.5, 500 mM NaCl; 5% Glycerol; 0.5 mM TCEP.

Column 3 Procedure: Total 5mg of protein was cleaved with 300µg of TEV protease at 4°C for 48 hours.

Column 4: HP Q column(ion exchange).

Column 4 Buffers:
Buffer A: 20 mM Tris-Cl, pH 8.5; 50 mM NaCl.
Buffer B: 20 mM Tris-Cl, pH 8.5; 2 M NaCl.

Column 4 Procedure: The target protein was applied to 5ml of HP Q column in buffer and eluted from the column by a linear gradient with buffer B.

Protein concentration: The target protein was concentrated to 10mg/ml using VivaSpin 5K concentrators and stored at -80°C.

Mass spectrometry characterization: Corresponds to theoretical mass, as determined by ESI-TOF MS.

Crystallisation: Crystals were grown by vapour diffusion in sitting drops at 4°C. Before setting up the experiment NAD^+ was added to the protein to a final concentration of 5mM. A sitting drop consisting of 75nl protein and 75nl well solution (20% PEG 3350, 0.2M Na_2SO_4, 10% ethylene glycol, 0.1M Bis-Tris Propane pH 6.5) was equilibrated against well solution. Crystals were cryo protected in 22.5% glycerol and flash-cooled in liquid nitrogen.

Data Collection:
Resolution: 2.15 Å.
X-ray source: Rigaku FRE Superbright.