Entry Clone Source: MGC

Entry Clone Accession: IMAGE:5266439

SGC Construct ID: HDHD2A-c100

GenBank GI number: gi|14149777

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

Amplified construct sequence:

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CTTAAGAAGGAGATATACTATGGCAGCAT
GCCGTGCATTAAAAGCTGTTTTGGTAGAT
CTCAGTGGCACACTTCACATTGAAGATGC
AGCTGTGCCAGGCGCACAGGAAGCTCTTA
AAAGGTTACGTGGTGCTTCTGTAATCATT
AGGTTTGTGACCAATACAACCAAAGAGAG
CAAGCAAGACCTGTTAGAAAGGTTGAGAA
AATTGGAATTTGATATCTCTGAAGATGAA
ATATTCACATCTCTGACTGCAGCCAGAAG
TTTACTAGAGCGGAAACAAGTCAGACCCA
TGCTGCTAGTTGATGATCGGGCACTACCT
GATTTCAAAGGAATACAAACAAGTGATCC
TAATGCTGTGGTCATGGGATTGGCACCAG
AACATTTTCATTATCAAATTCTGAATCAA
GCATTCCGGTTACTCCTGGATGGAGCACC
TCTGATAGCAATCCACAAAGCCAGGTATT
ACAAGAGGAAAGATGGCTTAGCCCTGGGG
CCTGGACCATTTGTGACTGCTTTAGAGTA
TGCCACAGATACCAAAGCCACAGTCGTGG
GGAAACCAGAGAAGACGTTCTTTTTGGAA
GCATTGCGGGGCACTGGCTGTGAACCTGA
GGAGGCTGTCATGATAGGAGATGATTGCA
GGGATGATGTTGGTGGGGCTCAAGATGTC
GGCATGCTGGGCATCTTAGTAAAGACTGG
GAAATATCGAGCATCAGATGAAGAAAAAA
TTAATCCACCTCCTTACTTAACTTGTGAG
AGTTTCCCTCATGCTGTGGACCACATTCT
GCAGCACCTATTGGCAGAGAACCTCTACT
TCCAATCGCACCATCATCACCACCATGAT
TACAAGGATGACGACGATAAGTGAGGATC
C
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Tags and additions: C-terminal Histidine-tag with TEV protease cleavage site

Final protein sequence (tag sequence in lowercase):

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MAACRALKAVLVDLSGTLHIEDAAVPGAQ
EALKRLRGASVIIRFVTNTTKESKQDLLE
RLRKLEFDISEDEIFTSLTAARSLLERKQ
VRPMLLVDDRALPDFKGIQTSDPNAVVMG
LAPEHFHYQILNQAFRLLLDGAPLIAIHK
ARYYKRKDGLALGPGPFVTALEYATDTKA
TVVGKPEKTFFLEALRGTGCEPEEAVMIG
DDCRDDVGGAQDVGMLGILVKTGKYRASD
EEKINPPPYLTCESFPHAVDHILQHLLae
nlyfqshhhhhhdykddddk
```

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.

Extraction buffer, extraction method: Lysis buffer: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 5 mM Imidazole, Complete® protease inhibitors (Roche, 1 tbl/50 ml). Frozen cell pellets were thawed and resuspended in a total volume of 30-40 ml 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

Buffers: Lysis/binding buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 5 mM Imidazole; **Wash buffer:** 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 5% Glycerol, 30 mM Imidazole; **Elution buffer:** 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole. **Note:** All the buffers contain 0.5mM TCEP.

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

Column 2: SuperDex 200 16/60 HiLoad (GE/Amersham)

Buffer: 10 mM HEPES, pH 7.5, 500 mM NaCl, 5 % glycerol, 0.5 mM TCEP.

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

Enzymatic treatment: TEV cleaved.

Column 3: Ni-Sepharose 6 Fast Flow (TEV clean up)

Buffer: 10 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP

Procedure: Total 5 mgs of protein was cleaved with 300 ug of TEV protease at 4°C for 48 hours.

TEV clean up: The TEV cleaved protein was applied to a 1 ml Ni-Sepharose 6 Fast Flow column, pre-equalibrated with gel filtration buffer (10 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP). The flow through from the column was collected. The eluate from the column was monitored by SDS gel analysis.

Concentration: The target protein (in buffer; 10 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP) was concentrated to 18.56 mg/ml using Vivaspin 30K concentrators and stored at -80°C.

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

Crystallization: Crystals were grown by vapour diffusion at 20°C. Before setting up the experiment substilisin was added to the protein at an enzyme:protein ratio of 1:500. A hanging drop consisting of 50 nl protein and 100 nl well solution was equilibrated against well solution containing 2M Ammonium sulphate, 1% MPD, 0.1M Hepes pH: 7.5. Crystals were cryo protected in sucrose and flash-cooled in liquid nitrogen.

Data Collection, Resolution: 2.5 Å **, X-ray source:** Synchrotron SLS-X10SA, single wavelength.