Entry Clone Source: MGC

Entry Clone Accession: IMAGE:2820849

SGC Construct ID: GOT1A-c110

GenBank GI number: gi|4504067

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

Amplified construct sequence:

CTTAAGAAGGAGATATACTATGCAGCCTGT
CCTGGTCTTCAAGCTCACTGCCGACTTCAG
GGAGGATCCGGACCCCCGCAAGGTCAACCT
GGGAGTGGGAGCATATCGCACGGATGACTG
CCATCCCTGGGTTTTGCCAGTAGTGAAGAA
AGTGGAGCAGAAGATTGCTAATGACAATAG
CCTAAATCACGAGTATCTGCCAATCCTGGG
CCTGGCTGAGTTCCGGAGCTGTGCTTCTCG
TCTTGCCCTTGGGGATGACAGCCCAGCACT
CAAGGAGAAGCGGGTAGGAGGTGTGCAATC
TTTGGGGGGAACAGGTGCACTTCGAATTGG
AGCTGATTTCTTAGCGCGTTGGTACAATGG
AACAAACAACAAGAACACACCTGTCTATGT
GTCCTCACCAACCTGGGAGAATCACAATGC
TGTGTTTTCCGCTGCTGGTTTTAAAGACAT
TCGGTCCTATCGCTACTGGGATGCAGAGAA
GAGAGGATTGGACCTCCAGGGCTTCCTGAA
TGATCTGGAGAATGCTCCTGAGTTCTCCAT
TGTTGTCCTCCACGCCTGTGCACACAACCC
AACTGGGATTGACCCAACTCCGGAGCAGTG
GAAGCAGATTGCTTCTGTCATGAAGCACCG
GTTTCTGTTCCCCTTCTTTGACTCAGCCTA
TCAGGGCTTCGCATCTGGAAACCTGGAGAG
AGATGCCTGGGCCATTCGCTATTTTGTGTC
TGAAGGCTTCGAGTTCTTCTGTGCCCAGTC
CTTCTCCAAGAACTTCGGGCTCTACAATGA
GAGAGTCGGGAATCTGACTGTGGTTGGAAA
AGAACCTGAGAGCATCCTGCAAGTCCTTTC
CCAGATGGAGAAGATCGTGCGGATTACTTG
GTCCAATCCCCCCGCCCAGGGAGCACGAAT
TGTGGCCAGCACCCTCTCTAACCCTGAGCT
CTTTGAGGAATGGACAGGTAATGTGAAGAC
AATGGCTGACCGGATTCTGACCATGAGATC
TGAACTCAGGGCACGACTAGAAGCCCTCAA
AACCCCTGGGACCTGGAACCACATCACTGA
TCAAATTGGCATGTTCAGCTTCACTGGGTT
GAACCCCAAGCAGGTTGAGTATCTGGTCAA
TGAAAAGCACATCTACCTGCTGCCAAGTGG
TCGAATCAACGTGAGTGGCTTAACCACCAA
AAATCTAGATTACGTGGCCACCTCCATCCA
TGAAGCAGTCACCAAAATCGCAGAGAACCT
CTACTTCCAATCGCACCATCATCACCACCA
TGATTACAAGGATGACGACGATAAGTGAGG
ATCC

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

Expressed sequence (tag sequence in lowercase):

MQPVLVFKLTADFREDPDPRKVNLGVGAYR

TDDCHPWVLPVVKKVEQKIANDNSLNHEYL
PILGLAEFRSCASRLALGDDSPALKEKRVG
GVQSLGGTGALRIGADFLARWYNGTNNKNT
PVYVSSPTWENHNAVFSAAGFKDIRSYRYW
DAEKRGLDLQGFLNDLENAPEFSIVVLHAC
AHNPTGIDPTPEQWKQIASVMKHRFLFPFF
DSAYQGFASGNLERDAWAIRYFVSEGFEFF
CAQSFSKNFGLYNERVGNLTVVGKEPESIL
QVLSQMEKIVRITWSNPPAQGARIVASTLS
NPELFEEWTGNVKTMADRILTMRSELRARL
EALKTPGTWNHITDQIGMFSFTGLNPKQVE
YLVNEKHIYLLPSGRINVSGLTTKNLDYVA
TSIHEAVTKIaenlyfqshhhhhhdykddd
dk

Tag sequence: aenlyfq(*)shhhhhhdykddddk

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

Expression protocol: 10µl of BL21(DE3)-R3-pRARE2 glycerol stock were inoculated into 5ml of TB with 50µg/ml of kanamycin and 34µg/ml chloramphenicol and grown overnight at 37°C, 200rpm. 10ml of overnight culture were added to 1L of TB with 50µg/ml kanamycin and incubated at 37°C, 160rpm. After the OD_{600} reached 1.0, the temperature was dropped to 18°C and 500µl 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.

Cell 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.

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

Solutions:
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 extinction at 280nm was monitored and fractions were collected and analyzed by SDS-PAGE. Positive fractions were pooled and characterised by the mass spec

Concentration and buffer exchange:
Using MilliPore concentrators with 30 kDa cutoff, the sample was concentrated to 24mg/ml. Concentrations were determined from the absorbance at 280 nm using NanoDrop.

Mass spectrometry characterization : ESI-MS revealed that the protein had a mass of 47696 Da (expected mass 47710 Da), indicating a difference of 14 Da.

Crystallization: Crystals were grown by vapor diffusion at 4°C in 150nl sitting drops. PLP (Pyridoxal phosphate) was added to 24mg/ml protein solution to a final concentration of 2 mM prior to crystallisation. The drops were prepared by mixing 50nl of protein solution and 100nl of precipitant consisting of 0.25 M NaK tartrate and 15w/v PEG 3350. Crystals were transferred to a cryo-protectant consisting of 20% Ethylene Glycol and 90% well solution before flash-cooling in liquid nitrogen

Data Collection:Resolution: 2.05Å; **X-ray source:**Diamond I03