Entry Clone Source: Site-directed mutagenesis Thr113Ala (underlined below)

Entry Clone Accession: n/a

SGC Construct ID: UGDHA-c704

GenBank GI number: gi|4507813

Vector: pBEN1-SGC. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

CATATGGACCCCGAAGAGGCGAGTGTTACT
TCAACAGAAGAAACCTTAACGCCAGCACAG
GAGGCCGCACGCACCCGCGCTGCTAACAAA
GCCCGAAAGGAAGCTGAGTTGGCTGCTGCC
ACCGCTGAACAAACTAGTGACGAGAAGACC
ACCGGCTGGCGGGGCGGCCACGTGGTGGAG
GGCCTGGCCGGCGAGCTGGAGCAGCTGCGG
GCCAGGCTGGAGCACCACCCTCAGGGCCAG
CGGGAGCCCTCCGGCGGCTGCAAGCTGGGC
CTGGGTACCGAGAACCTGTACTTCCAATCC
ATGTTTGAAATTAAGAAGATCTGTTGCATT
GGTGCAGGCTATGTTGGAGGACCCACATGT
AGTGTCATTGCTCATATGTGTCCTGAAATC
AGGGTAACGGTTGTTGATGTCAATGAATCA
AGAATCAATGCGTGGAATTCTCCTACACTT
CCTATTTATGAGCCAGGACTAAAAGAAGTG
GTAGAATCCTGTCGAGGAAAAAATCTTTTT
TTTTCTACCAATATTGATGATGCCATCAAA
GAAGCTGATCTTGTATTTATTTCTGTGAAT
ACTCCAACAAAAACCTATGGAATGGGGAAA
GGCCGGGCAGCAGATCTGAAGTATATTGAA
GCTTGTGCTAGACGCATTGTGCAAAACTCA
AATGGGTACAAAATTGTGACTGAGAAAAGC
GCAGTTCCGGTGCGGGCAGCAGAAAGTATC
CGTCGCATATTTGATGCAAACACAAAACCC
AACTTGAATTTACAGGTGCTGTCCAACCCT
GAGTTTCTGGCAGAGGGAACAGCCATCAAG
GACCTAAAGAACCCAGACAGAGTACTGATT
GGAGGGGATGAAACTCCAGAGGGCCAGAGA
GCTGTGCAGGCCCTGTGTGCTGTATATGAG
CACTGGGTTCCCAGAGAAAAGATCCTCACC
ACTAATACTTGGTCTTCAGAGCTTTCCAAA
CTGGCAGCAAATGCTTTTCTTGCCCAGAGA
ATAAGCAGCATTAACTCCATAAGTGCTCTG
TGTGAAGCAACAGGAGCTGATGTAGAAGAG
GTAGCAACAGCGATTGGAATGGACCAGAGA
ATTGGAAACAAGTTTCTAAAAGCCAGTGTT
GGGTTTGGTGGGAGCTGTTTCCAAAAGGAT
GTTCTGAATTTGGTTTATCTCTGTGAGGCT
CTGAATTTGCCAGAAGTAGCTCGTTATTGG
CAGCAGGTCATAGACATGAATGACTACCAG
AGGAGGAGGTTTGCTTCCCGGATCATAGAT
AGTCTGTTTAATACAGTAACTGATAAGAAG
ATAGCTATTTTGGGATTTGCATTCAAAAAG
GACACTGGTGATACAAGAGAATCTTCTAGT
ATATATATTAGCAAATATTTGATGGATGAA
GGTGCACATCTACATATATATGATCCAAAA
GTACCTAGGGAACAAATAGTTGTGGATCTT

TCTCATCCAGGTGTTTCAGAGGATGACCAA
GTGTCCCGGCTCGTGACCATTTCCAAGGAT
CCATATGAAGCATGTGATGGTGCCCATGCT
GTTGTTATTTGCACTGAGTGGGACATGTTT
AAGGAATTGGATTATGAACGCATTCATAAA
AAAATGCTAAAGCCAGCCTTTATCTTCGAT
GGACGGCGTGTCCTGGATGGGCTCCACAAT
GAACTACAAACCATTGGCTTCCAGATTGAA
ACAATTGGCAAAAAGGTGTGACAGTAAAGG
TGGATACGGATCCGAATTCGAGCTCCGTCG
ACAAGCTTGCGGCCGCACTCGAGCACCACC
ACCACCACCACTGA

Final protein sequence (tag sequence in lowercase):
mdpeeasvtsteetltpaqeaartraanka
rkeaelaaataeqtsdekttgwrgghvveg
lageleqlrarlehhpqgqrepsggcklgl
gtenlyfq*sMFEIKKICCIGAGYVGGPTC
SVIAHMCPEIRVTVVDVNESRINAWNSPTL
PIYEPGLKEVVESCRGKNLFFSTNIDDAIK
EADLVFISVNTPTKTYGMGKGRAADLKYIE
ACARRIVQNSNGYKIVTEKS***A***VPVRAAESI
RRIFDANTKPNLNLQVLSNPEFLAEGTAIK
DLKNPDRVLIGGDETPEGQRAVQALCAVYE
HWVPREKILTTNTWSSELSKLAANAFLAQR
ISSINSISALCEATGADVEEVATAIGMDQR
IGNKFLKASVGFGGSCFQKDVLNLVYLCEA
LNLPEVARYWQQVIDMNDYQRRRFASRIID
SLFNTVTDKKIAILGFAFKKDTGDTRESSS
IYISKYLMDEGAHLHIYDPKVPREQIVVDL
SHPGVSEDDQVSRLVTISKDPYEACDGAHA
VVICTEWDMFKELDYERIHKKMLKPAFIFD
GRRVLDGLHNELQTIGFQIETIGKKV

Tags and additions: N-terminal SET1 and SBP tags, followed by a TEV protease cleavage site:
mdpeeasvtsteetltpaqeaartraanka
rkeaelaaataeqtsdekttgwrgghvveg
lageleqlrarlehhpqgqrepsggcklgl
gtenlyfq*s (* - TEV cleavage site)
Tag removed: yes

Host: BL21(DE3)-R3-pRARE2

Expression protocol: A glycerol stock of host strain BL21(DE3)-R3-pRARE2 carrying the expression plasmid was used to inoculate 10 ml of TB (terrific Broth) supplemented with 50 µg/ml kanamycin and 34 µg/ml chloramphenicol. This starter culture was grown overnight at 37°C and used to inoculate 6X 1 liter culture in the same media (initial OD_{600}= 0.01). The culture was grown at 37°C until the OD_{600} reached ˜0.8. After that the temperature was lowered to 18°C and protein production was induced with 0.2 mM IPTG. Recombinant UGDHA was expressed at that temperature overnight. The next day cells were harvested by centrifugation at 4000 rpm for 30 minutes and the pellet was resuspended in 150 ml Strepavidin binding buffer supplemented with Complete Protease Inhibitors (1 tablet/50 ml) and stored at -20°C.

Cell extraction:
Strepavidin binding buffer: 20 mM Tris, pH 8.0, 150 mM NaCl.
Procedure: Frozen cell pellets were thawed and lysed by passing 5 times through a high pressure homogenizer. The lysate was clarified by centrifuging for 60 minutes at 21,000 rpm at 4C. Before applying to the column the laysate was further clarified by passing through a 1.2µm syringe filter

Column 1: Streptavidin sepharose

Column 1 Buffers: Binding and washing buffer: 20 mM Tris, pH 8.0, 150 mM NaCl; **Elution buffer:** 20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 2mM Biotin.

Column 1 Procedure: Ten ml Streptavidin sepharose resin was equilibrated with 100 mL of binding buffer. The supernatant was incubated with the washed resin at 4°C for 2 hour by gentle rotation and packed into the column. After the lysate had passed through the column it was washed with 60 ml of washing buffer. The protein was eluted with 30 ml of elution buffer. The eluted protein was concentrated (Vivaspin centricon MWCO 30kDa) and exchanged into gel filtration buffer using a PD-10 desalting column.

Enzymatic treatment : His-tagged TEV protease was added to the protein using a 1:20 TEV to protein ratio (mg/mg). The digestion was incubated 48 hours at 4°C.

Column 2: Gel filtration, Hiload 16/60 Superdex S200 prep grade, 120 ml (GE Healthcare)

Column 2 Buffer: **Gel Filtration Buffer** - 50mM HEPES pH 7.5, 300mM NaCl, 0.5 mM TCEP

Column 2 Procedure: Following TEV digestion, the protein sample was loaded onto an S200 column at 1 ml/min using an AKTA Purifier system at 4°C. Fractions were analysed by SDS - PAGE and pooled according to purity. The eluted protein was concentrated (Vivaspin centricon MWCO 30kDa) and buffer exchanged into ion exchange buffer A using a PD-10 desalting column.

Column 3: MonoQ 5/50 (Qiagen)

Column 3 Buffers: Buffer A: 50 mM HEPES, pH 7.5; **Buffer B:** 50 mM HEPES, pH 7.5, 1 M NaCl

Column 3 Procedure: The protein was applied to the column in Buffer A and eluted using a linear gradient from 0 - 50% Buffer B. After SDS-Page analysis of the fractions, pure protein was pooled and concentrated using a Vivaspin centricon with a MWCO of 30kDa.

Mass spectrometry characterization: ESI-MS revealed that the protein had a mass of 51991 Da (Expected mass 51990 Da).

Crystallization: Crystals were grown at 20°C by vapour diffusion in sitting drops by mixing protein (20 mg/ml) and well solution containing 15% PEG smear, 0.1M MES, pH 6.0 at a protein to precipitant ratio of 1:1. A crystal was cryo-protected using well solution supplemented with 20% (v/v) ethylene glycol and flash-cooled in liquid nitrogen.

Data Collection:
Resolution (scaled): 2.4 Å
X-ray source: Diamond light source I03.