Entry Clone Source: Origene

Entry Clone Accession: NM_182617 Variant

SGC Construct ID: LOC148158A-c593

GenBank GI number: gi|32699052

Vector: pFBOH-LIC-Bse

Tags and additions: N-terminal, TEV cleavable hexahistidine tag. Tag sequence:
mhhhhhhssgvdlgtenlyfq(*)sm

Host:*Trichoplusia Ni* (High five)

Final protein sequence:
mghhhhhhssgvdlgtenlyfqsMSLQWG
HQEVPAKFNFASDVLDHWADMEKAGKRPP
SPALWWVNGKGKELMWNFRELSENSQQAA
NVLSGACGLQRGDRVAVVLPRVPEWWLVI
LGCIRAGLIFMPGTIQMKSTDILYRLQMS
KAKAIVAGDEVIQEVDTVASECPSLRIKL
LVSEKSCDGWLNFKKLLNEASTTHHCVET
GSQEASAIYFTSGTSGLPKMAEHSYSSLG
LKAKMDAGWTGLQASDIMWTISDTGWILN
ILCSLMEPWALGACTFVHLLPKFDPLVIL
KTLSSYPIKSMMGAPIVYRMLLQQDLSSY
KFPHLQNCVTVGESLLPETLENWRAQTGL
DIRESYGQTETGLTCMVSKTMKIKPGYMG
TAASCYDVQIIDDKGNVLPPGTEGDIGIR
VKPIRPIGIFSGYVDNPDKTAANIRGDFW
LLGDRGIKDEDGYFQFMGRADDIINSSGY
RIGPSEVENALMEHPAVVETAVISSPDPV
RGEVVKAFVVLASQFLSHDPEQLTKELQQ
HVKSVTAPYKYPRKIEFVLNLPKTVTGKI
QRAKLRDKEWKMSGKARAQ

Expression: High five cells were grown in Sf900II medium supplemented with 1% FCS at 27°C. Cells were infected at a density of 2x106/ml with recombinant baculovirus (virus stock P2; 1ml of virus stock/1l of cell culture). 72 hours post-infection the cultures were collected and centrifuged for 30min at 2000rpm. The cell pellet was resuspended in cold PBS and centrifugation was repeated. The pellet was resuspended in lysis buffer (50mM HEPES pH 7.5, 500mM NaCl, 5mM Imidazole, 5% glycerol + EDTA-free Complete (1 tablet/50ml)) and frozen at -80°C till purification.

Extraction: Lysis buffer: 50mM HEPES pH 7.5, 500mM NaCl, 5mM Imidazole, 5% glycerol + EDTA-free Complete (1 tablet/50ml). The thawed cellular lysates were supplemented with Igepal CA 630 (Fluka) at a final concentration of 0.05%, and benzonase (Novagen) (25u/10ml of lysate). Cells were broken using a Dounce homogeniser, followed by centrifugation for 45 min at 20.000rpm at 4°C.

Purification:
Step 1: Ni-affinity, Ni-Sepharose - (GE Healthcare) purification in batch
Step 2: Superdex 200Column , HiPrep 16/60 (Amersham)
Step 3: Ion exchange -5ml HiTrap Q Sepharose

Buffers:
Start buffer: 50mM HEPES pH 7.5, 500mM NaCl, 5mM Imidazole, 5% glycerol, 1mM PMSF, 0.5mM TCEP
Washing buffer: 50mM HEPES pH 7.5, 500mM NaCl, 10mM 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
IEX buffers: **Buffer A:** 50mM Tris pH 9.0
Buffer B: 50mM Tris pH 9.0, 1M NaCl

Procedure: The cell extract was incubated with Ni-Sepharose (0.250µl of resin/lysate from 1l of culture) during 1h at 4°C with gentle rotation. The resin was centrifuged at 1000g for 5min at 4°C, and washed 4x with 50ml of washing buffer. Resin was loaded on the gravity column and protein was eluted in 4 elution fractions (5ml each). Protein fractions were analysed by SDS-PAGE. Target protein containing fractions were concentrated using Amicon Ultra-15 concentrators with 10kDa cut-off, and purified on a gel filtration column (Superdex 200) on an Akta Purifier System. LOC148158 containing fractions were diluted with IEX buffer A to a concentration of NaCl of 50 mM. The protein was loaded onto a HiTrap Q Sepharose column, and eluted with a NaCl gradient (50-300mM). Fractions containing protein were analysed by SDS-PAGE.

Concentration and buffer exchange: Using Amicon Ultra-15 concentrators with 10kDa cutoff, the sample was concentrated to 20mg/ml. Concentrations were determined from the absorbance at 280 nm using a NanoDrop spectrophotometer.

Mass spec characterization: The calculated mass of the construct was 63260Da, and the observed mass (ESI-MS) was 63173Da, compatible with an N-terminal methionine deletion and subsequent acetylation at the N-teminus of the protein.

Crystallization: Crystals were grown by vapor diffusion at 20°C in 150nl sitting drops. Butyryl coA and AMP were added to the protein solution before crystallization to a final concentration of 5mM. The drops were prepared by mixing 75nl of protein solution and 75nl of precipitant consisting of 0.1M Mg(form)2 15% PEG 3350. Crystals were flash-cooled in liquid nitrogen with 25 % glycerol as cryoprotectant.

Data Collection: Resolution: 2.5Å; **X-ray source:** rotating anode (Rigaku FR-E SuperBright), single wavelength.