

ACOT7

PDB:2QQ2

Revision

Revision Type:created

Revised by:created

Revision Date:created

Entry Clone Accession:BC017365

Entry Clone Source:Mammalian Gene Collection

SGC Clone Accession:

Tag:N-terminal hexahistidine tag with integrated TEV protease cleavage site:

mhhhhhssgvdlgtenlyfq*sm

Host:*E.coli* BL21-Gold(DE3)pRARE2, where BL21-Gold(DE3) cells (Stratagene) have been transformed with pRARE2 originating from the Rosetta2 strain (Novagen). The pRARE2 plasmid supplies tRNAs for rare codons.

Construct

Prelude:

Sequence:

mhhhhhssgvdlgtenlyfq*smGDIVQPVLNPEPNTVSYSQSSLIHLVGPSDCTLHGFBVHGGVTMKLMDEVAGIVAARHCKTNIV
TASVDAINFHDKIRKGCVITISGRMTFTSNKSMEIEVLVDADPVVDSSQKRYRAASAFFTYVSLSQEGRSLPVPQLVPETEDKKRF
EEGKGRLQMKAKRQGHAEF

Vector:pNIC-Bsa4

Growth

Medium:

Antibiotics:

Procedure:Cells from a glycerol stock were grown in 20 ml TB supplemented with 8 g/l glycerol, 100 µg/ml kanamycin and 34 µg/ml chloramphenicol at 30 °C overnight. The overnight culture (20 ml) was used to inoculate 1.5 l TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin and approximately 200 µl BREOX FMT 30 anti-foam solution (Cognis Performance Chemicals UK Ltd). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C until OD600 reached ~2. The culture was down-tempered to 18 °C over a period of 1 hour before target expression was induced by addition of 0.5 mM IPTG. Expression was allowed to continue overnight and cells were harvested the following morning by centrifugation (5,500 x g, 10 min, 4 °C). The resulting cell pellet (27.1 g wet cell weight) was resuspended in lysis buffer (2 ml/g cell pellet), supplemented with one tablet of Complete EDTA-free protease inhibitor (Roche Applied Science). The cell suspension was stored at -80 °C.

Purification

Procedure

Columns

IMAC: Ni-charged 1 ml HiTrap Chelating HP (GE Healthcare)

Gel filtration column: HiLoad 16/60 Superdex 75 Prep Grade (GE Healthcare)

Procedure

Purification of the protein was performed as a two step process on an ÄKTAexpress system (GE Healthcare). Prior to purification, columns were equilibrated with IMAC wash1 buffer and gel filtration buffer, respectively. The filtered lysate was loaded onto the Ni-charged HiTrap Chelating column and washed with IMAC wash1 buffer followed by IMAC wash2 buffer. Bound protein was eluted from the IMAC column with IMAC elution buffer and automatically loaded onto the gel filtration column. The chromatogram from gel filtration showed one major protein peak. Fractions containing the target protein were pooled and fresh TCEP was added to a final concentration of 2 mM. The protein was subsequently concentrated using an Amicon Ultra-15 centrifugal filter device, 10,000 NMWL (Millipore) to 37.1 mg/ml in a volume of 1.1 ml. The identity of the protein was confirmed by mass spectrometry.

Extraction

Procedure

The cell suspension was quickly thawed in water and 2000 U Benzonase (Merck) was added. Cells were disrupted by sonication (Vibra-Cell, Sonics) at 80% amplitude for 3 min effective time (pulsed 4s on, 4s off) and cell debris was removed by centrifugation (49,100 x g, 20 min, 4 °C). The supernatant was decanted and filtered through a 0.45 µm flask filter.

Concentration:

Ligand

MassSpec:

Crystallization: Crystals were obtained by the hanging drop vapour diffusion method in a 24-well plate containing 500 µl well solution. 1.2 µl of the protein solution (37 mg/ml) was mixed with 0.6 µl of well solution consisting of 0.1 M bis-Tris pH 6.0, 0.2 M MgCl₂ and 21% PEG 3350. The plate was incubated at 20 °C and crystals appeared within one month. The crystals were quickly transferred to cryo solution containing paratone and flash frozen in liquid nitrogen.

NMR Spectroscopy:

Data Collection: Resolution: 2.8 Å; X-ray source: ESRF Beamline 14-2, single wavelength.

Data Processing: Indexed and scaled with XDS. Refined with PHENIX and REFMAC5.