Entry Clone Source: IMAGE

Entry Clone Accession: IMAGE:40017263

SGC Construct ID: MMAAA-c301

GenBank GI number: gi|26892295

Vector: pNIC28-Bsa4. Details [PDF]; Sequence [FASTA] or [GenBank]

Tags and additions: N-terminal, TEV cleavable hexahistidine tag

Final protein sequence:

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sMKDHTEGLSDKEQRFVDKLYTGLIQGQR
ACLAEAITLVESTHSRKKELAQVLLQKVL
LYHREQEQSNKGKPLAFRVGLSGPPGAGK
STFIEYFGKMLTERGHKLSVLAVDPSSCT
SGGSLLGDKTRMTELSRDMNAYIRPSPTR
GTLGGVTRTTNEAILLCEGAGYDIILIET
VGVGQSEFAVADMVDMFVLLLPPAGGDEL
QGIKRGIIEMADLVAVTKSDGDLIVPARR
IQAEYVSALKLLRKRSQVWKPKVIRISAR
SGEGISEMWDKMKDFQDLMLASGELTAKR
RKQQKVWMWNLIQESVLEHFRTHPTVREQ
IPLLEQKVLIGALSPGLAADFLLKAFKSR
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Expression strain: BL21(DE3)-R3-pRARE2 (previously known as Rosetta)

Transformation: The construct DNA was transformed into homemade chemically competent cells of the expression strain by a standard heat shock procedure.

Glycerol stock prepataion: A number of colonies from the transformation were used to inoculate 1 ml of LB media containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day glycerol stocks were prepared from this overnight culture.

Expression: 10 ml of a glycerol stock was used to inoculate 40 ml of LB media containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol, which was placed in a 37°C shaker overnight.

The next day, the starter culture was used to inoculate TB media (11 ml starter culture per litre) containing 50 µg/ml kanamycin. At OD~1.8, the cells were induced by the addition of 0.5 mM IPTG. The expression was continued overnight at 37°C (~18 hours).

Cell harvest: Cells were spun at 6238x g for 15 mins at 4°C. The yield was 8g cells / litre culture. The pellets were placed in a -80°C freezer.

Cell Lysis: The pellets were resuspended in lysis / binding buffer. They were passed 4 times through an Emulsiflex C5 high-pressure homogeniser, collecting a final volume of approximately 20 ml/ litre culture . PEI was added to a final concentration of 0.25 % and the cell debris and precipitated DNA were spun down at 45000x g, 90 min (Beckman JA 18 17500 rpm). The supernatant was collected. **Lysis/ Binding Buffer:** 50mM HEPES pH 7.4, 500mM NaCl, 5% glycerol, 30 mM Imidazole pH 7.4, 0.5 mM TCEP, 1mM PMSF
Column 1: Ni-Sepharose gravity-flow column

Buffers:
Wash Buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 70 mM Imidazole pH 7.4, 0.5 mM TCEP
Elution Buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole pH 7.4, 0.5 mM TCEP

Procedure: The supernatant was loaded onto an equilibrated Ni- sepharose column (0.4 ml resin / litre culture). The flow through was collected .The column was first washed with 15CV of Lysis/Binding Buffer , followed by 7CV of Wash buffer and finally eluted with 6x1CV elution buffer . Each fraction was collected and separated on SDS-PAGE.
Column 2: Gel filtration. Hiload S200 16/60 - 120 ml volume.
Gel Filtration buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP
Procedure: The gel filtration column was pre-equilibrated with Gel Filtration Buffer. The Ni-Sepharose eluant was loaded on the gel filtration column at a flow rate of 1.2 ml/min. Eluted proteins were collected in 1.8 ml fractions. The fractions containing protein were identified by SDS-PAGE.
TEV protease digestion: The gel filtration fractions containing MMAAA were pooled and 45mg of TEV protease was added per mg protein. The digestion was left overnight at 4°C.
Rebinding of impurities to Ni-NTA: The protein was batch bound to Ni-sepharose resin at 4°C for 60 minutes. The resin was spun down and the supernatant was filtered through a 0.2 µm filter and collected .
Concentration: The TEV protease cleaved MMAAA was concentrated to 14.5 mg/ml , distributed into 70 ml aliquots and frozen at -80°C.
Mass spectrometry characterisation: Measured: 38665.1; **Expected:** 38663.9.
Crystallisation: Prior to crystallization, protein (10mg/ml) was pre-incubated with 1.5mM GMPPNP at 4°C. Crystals were grown by the vapour diffusion method at 20°C. Sitting drops mixing 50nl protein and 100nl well solution containing 32.5% v/v LMW PEG smear (PEG 300, 400, 550MME, 600, 1000), 0.2M ammonium nitrate and 0.1M sodium cacodylate pH 5.0 were equilibrated against reservoirs containing 20ul of well solution. Crystals were cryo-protected in 25% ethylene glycol before flash-cooling in liquid nitrogen.
Data Collection: Resolution: 2.64 Å; **X-ray source:** Synchrotron Diamond IO3