Entry Clone Source: Synthetic

Entry Clone Accession: n/a

SGC Construct ID: LOC390245C-c103

GenBank GI number: gi|89034221

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

Tag and Additions: N-Terminal TEV cleavable 6His tag - `mhhhhhhssgvdltgtenlyfq*s(m)`, TEV cleaves at *.

Protein Sequence (tag sequence in lowercase):
mhhhhhhssgvdlgtenlyfq*sMKSVHS
SPQNTSHTIMTFYPTMEEFADFNTYVAYM
ESQGAHQAGLAKVIPPKEWKARQMYDDIE
DILIATPLQQVTSGQGGVFTQYHKKKAM
RVGQYRRLANSKKYQTPPHQNFADLEQRY
WKSHPGNPPIYGADISGSLFEESTKQWNL
GHLGTILDLLEQECGVVIEGVNTPYLYFG
MWKTTFAWHTEDMDLYSINYLHFGEPKTW
YVVPPEHGQHLERLARELFPDISRGCEAF
LRHKVALISPTVLKENGIPFNCMTQEAGE
FMVTFPYGYHAGFNHGFNCAEAINFATPR
WIDYGKMASQCSCGESTVTFSMDPFVRIV
QPESYELWKHR

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

Growth Medium, induction protocol: Medium: Terrific Broth +50 µg/ml Kanamycin, +34 µg/ml chloramphenicol. 6 x 1 litres TB in 2.5 L baffled flasks, each flask was inoculated with 5 ml overnight culture and grown at 37°C. The protein expression was induced with 0.2 mM IPTG at OD_{600}= 0.8 for 18 hours at 18°C. The cells were harvested by centrifugation, resuspended inlysis buffer (see below) and frozen at -80°C.

Lysis buffer, lysis method: Lysis Buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 20 mM Imidazole, 5% Glycerol, 0.5 mM TCEP, Complete Protease Inhibitor (Roche Biochemicals). Frozen cell pellets were thawed and lysed by high pressure homogenisation (15kpsi), viscosity was reduced by 3 further passes through the homogeniser. Cell debris were removed by centrifugation at 40,000 x g for 60 minutes. The lysate was then diluted 1:1 with Lysis buffer

All chromatography performed at 4°C.

Column 1: 5 ml bed volume Ni Sepharose 6 FF gravity column (GE/Amersham Biosciences)

Procedure: The gravity column was pre-equilibrated with IMAC Binding Buffer (Lysis Buffer without Complete PI), the lysate was applied to the column and allowed to flow through completely. The column was then washed with 2 x 10 ml of IMAC BInding Buffer. The column was then washed with 4 x 50 ml of IMAC Wash Buffer (IMAC Binding Buffer with 40 mM Imidazole). The column was then eluted with IMAC Elution Buffer (IMAC Binding Buffer with 250 mM Imidazole).

TEV digest: TEV protease was added to the eluated protein and incubated overnight at 4°C.

Column 2: HiPrep 26/1 Desalting

Procedure: The protein was then desalted into IMAC Binding Buffer using a HiPrep 26/10 desalting column on an AKTAPrime (GE/Amersham Biosciences).

Column 3: 2 ml bed volume Ni Sepharose 6 FF gravity column (GE/Amersham Biosciences)

Procedure: The protein was then applied to the column and the flow through collected. The column was washed with 2 x 5 ml of IMAC Binding Buffer and the flow through collected. The flow through was concentrated to 5 ml.
Column 4: HiLoad 16/60 SuperDex 200 Prep Grade
Procedure: The gel filtration column was equilibrated with 10 mM HEPES pH7.5, 500 mM NaCl, 5% Glycerol. The protein was applied to column and fractions collected. Fractions were analysed by SDS-PAGE, fractions were pooled based on SDS-PAGE purity. TCEP-HCl was added to 0.5 mM, slight precipitation was seen, the protein was centrifuged to remove precipitate and was immediately desalted.
Column 5: HiPrep 26/1 Desalting
Procedure: The desalting column was equilibrated with 10 mM HEPES pH7.5, 500 mM NaCl, 5% Glycerol. No TCEP-HCl added.
Concentration: The protein was concentrated to 10 mg/ml by A280 with a Millipore/Amicon Ultracel 30 kD MWCO ultrafiltration concentrator.
Mass spectrometry characterisation: The mass determined for the protein was 38681 Da, in agreement for the predicted mass for the cleaved protein.
Crystallisation: Crystals were grown by vapour diffusion at 4°C. 25 µl of protein had 2.5 µl of 25 mM 2,4 pyridine dicarboxylic acid added in 50 mM HEPES pH 7.5 added. A sitting drop consisting of 134 nl of protein + 66 nl of well solution was equilibrated against 0.1 M citrate pH 5.5, 20% PEG3350, 2 mM NiCl2. Cryo-protection of the crystals used 0.1 M citrate pH 5.5, 20% PEG3350, 2 mM NiCl2, 25% glycerol and 2.5 mM 2,4-PDCA.
Data Collection: Resolution: 2.1 Å; X-ray source: Synchrotron SLS-X10SA