Entry Clone Source: MGC
Entry Clone Accession: IMAGE:40003445
SGC Construct ID: CRYBA4A-c003
GenBank GI number: gi|4503059
Vector: pNIC28-Bsa4. Details [PDF]; Sequence [FASTA] or [GenBank]
E.coli strain: BL21(DE3)-R3 pRARE2
Plate: UO-106-pNIC28-Bsa4 (N-terminal His-tag)
Plate well ID: E7
Amplified construct sequence:

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CATATGCACCATCATCATCATCATTCTTCT
GGTGTAGATCTGGGTACCGAGAACCTGTAC
TTCCAATCCATGTCAGCGGGACCCTGGAAG
ATGGTGGTGTGGGATGAGGACGGCTTCCAG
GGCCGGCGGCACGAGTTCACGGCCGAGTGC
CCCAGCGTGCTGGAGCTTGGCTTCGAGACT
GTGCGATCTTTGAAAGTGCTGAGTGGAGCG
TGGGTGGGCTTCGAGCATGCTGGCTTCCAA
GGGCAGCAGTACATTCTGGAACGAGGCGAA
TATCCAAGCTGGGATGCCTGGGGCGGCAAC
ACGGCCTACCCCGCCGAGAGGCTCACCTCC
TTCCGGCCTGCGGCCTGTGCTAACCACCGT
GACTCGAGGCTGACAATCTTCGAGCAAGAG
AACTTCCTGGGCAAGAAAGGAGAGCTGAGC
GATGACTATCCTTCCCTCCAGGCCATGGGA
TGGGAAGGCAATGAAGTAGGGTCCTTCCAC
GTCCACTCTGGGGCCTGGGTTTGCTCCCAG
TTTCCGGGCTACCGAGGATTTCAGTATGTG
CTGGAATGCGATCACCATTCCGGTGACTAC
AAACATTTCCGGGAGTGGGGCTCTCATGCC
CCGACCTTCCAGGTGCAGAGCATCCGCAGG
ATCCAGCAGTGACAGTAAAGGTGGATACGG
ATCCGAA
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Tags and additions: N-terminal Histidine-tag with TEV protease cleavage site
Final protein sequence (tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfqsMSAGPWKM
VVWDEDGFQGRRHEFTAECPSVLELGFETV
RSLKVLSGAWVGFEHAGFQGQQYILERGEY
PSWDAWGGNTAYPAERLTSFRPAACANHRD
SRLTIFEQENFLGKKGELSDDYPSLQAMGW
EGNEVGSFHVHSGAWVCSQFPGYRGFQYVL
ECDHHSGDYKHFREWGSHAPTFQVQSIRRI
QQ
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Growth medium, induction protocol: 10ul of a glycerol stock was inoculated into 5ml of TB medium (supplemented with 50ug/ml Kanamycin, 34ug/ml Chloramphenicol) and cultured at 37°C o/n in a shaking incubator (250 rpm). Next day 0.75 ml of o/n culture was used to inoculate 1 litre of TB medium (6 x) and grown at 37°C with vigorous shaking (160 rpm) until the culture reaches an OD_{600} of 1.7. Temperature was reduced to 18°C, and cells were induced with IPTG at a concentration of 0.3 mM, and further cultivated for 16 hrs. Cells were harvested by centrifugation at 6500 rpm for 10 min, and the cell pellet was stored at -20°C until further use.

Extraction buffer, extraction method: Lysis buffer: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 5 mM Imidazole, Complete® protease inhibitors (Roche, 1 tbl/50 ml).
Frozen cell pellets were thawed and resuspended in a total volume of 30-40 ml of lysis buffer, and disrupted by using sonicator, and a supernatant containing the target protein was obtained by centrifugation at 21,000 rpm for 45 minutes.

Expression: 10ul of BL21(DE3)-R3-pRARE2 glycerol stock were inoculated into 5ml of TB with 50ug/ml of kanamycin and 34ug/ml chloramphenicol and grown overnight at 37°C, 200rpm. 10ml of overnight culture were added to 1L of TB with 50ug/ml kanamycin and incubated at 37°C, 160rpm. After the OD_{600} reached 1.0, the temperature was dropped to 18°C and 500ul of 1M IPTG was added to the final concentration of ~0.5mM. The culture was then incubated with shaking overnight at 18°C, 160rpm. The following morning the 4L culture was harvested and centrifuged for 10min at 4000rpm. Supernatant was discarded and cell pellets were resuspended in 80ml of a lysis buffer and frozen at -80°C.

Extraction: Lysis buffer: 50mM HEPES pH 7.5, 500mM NaCl, 5mM Imidazole, 5% glycerol + 1mM PMSF. The thawed cells were broken by 5 passes at 16.000 psi through a high pressure homogeniser followed by centrifugation for 45 min at 15.000rpm.

Column 1: Ni-Sepharose 6 Fast Flow

Column 1 Buffers: Lysis buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 5 mM Imidazole. **Wash buffer:** 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 5% Glycerol, 30 mM Imidazole. **Elution buffer:** 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole.
Note: All the buffers contain 0.5mM TCEP.

Column 1 Procedure: The column was packed with 2 ml of Ni-Sepharose 6 Fast Flow slurry and equilibrated with 15 ml of binding buffer. The supernatant was loaded onto the column and the column was washed with 20 ml of binding buffer and then 20 ml of washing buffer. The protein was eluted with 10 ml of elution buffer.

Column 2: SuperDex 75 16/60 HiLoad (GE Healthcare)

Column 2 Buffer: 10 mM HEPES, pH 7.5, 500 mM NaCl, 5 % glycerol, 0.5 mM TCEP.

Column 2 Procedure: The eluted protein from the Ni-affinity column was loaded on the gel filtration column in GF buffer at 1.0 ml/min on an AKTA Purifier system. Eluted proteins were collected in 1 ml fractions.

Enzymatic treatment: TEV cleaved.

Column 3: Ni-Sepharose (TEV clean up)

Column 3 Buffer: 10 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP

Column 3 Procedure: Total 5 mgs of protein was cleaved with 300 ug of TEV protease at 4° for 48 hours.

TEV clean up: The TEV cleaved protein was applied to a 1 ml Ni-Sepharose column, already pre-equilibrated with gel filtration buffer (10 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP). The flow through from the column was collected. The eluate from the column was monitored by SDS gel analysis.

Concentration: The target protein was concentrated to 17.94 mg/ml using Vivaspin 10K concentrators and stored at -80°C.

Mass spectrometry characterization: Corresponds to theoretical mass, as determined by ESI-TOF MS.

Crystallization: Crystals were grown by vapour diffusion in sitting drop at 20°C. A sitting drop consisting of 50 nl protein and 100 nl well solution was equilibrated against well solution containing 2M Ammonium-phosphate monobasic and 0.1M Tris-HCl pH 8.5. Crystals were cryo-protected in the presence of 20% (v/v) glycerol and flash-cooled in liquid nitrogen.

Data Collection: Resolution: 1.7 Å**, X-ray source:** Diamond Light Source, beamline I02 single wavelength.