Materials and Method

Note: To our best knowledge, this should represent an accurate description of the materials and methods required to reproduce our work. If any of the content on this page is difficult to interpret or should you have trouble repeating our work, do not hesitate to contact us as soon as possible in order for us to provide additional information and advice.

Entry Clone Source: Synthetic

Entry Clone Accession: n/a

SGC Construct ID: SEPX1A-c008

GenBank GI number: gi|7706511

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

Amplified construct sequence:

```
CATATGCACCATCATCATCATCATTCTTCT
GGTGTAGATCTGGGTACCGAGAACCTGTAC
TTCCAATCCATGGAGGTTTTCCAGAATCAC
TTTGAACCTGGCGTTTACGTGTGTGCCAAG
TGTGGCTATGAGCTGTTCTCCAGCCGCTCG
AAGTATGCACACTCGTCTCCATGGCCGGCG
TTCACCGAGACCATTCACGCCGACAGCGTG
GCCAAGCGTCCGGAGCACAATAGATCTGAA
GCCTTGAAGGTGTCCTGTGGCAAGTGTGGC
AATGGGTTGGGCCACGAGTTCCTGAACGAC
GGCCCCAAGCCGGGGCAGTCCCGATTCTCA
ATATTCAGCAGCTCGCTGAAGTTTGTCCCT
AAAGGCAAAGAAACTTCTGCCTGACAGTAA
AGGTGGATACGGATCCGAA
```

Tags and additions: N-terminal Histidine-tag with TEV protease cleavage site

Final protein sequence (tag sequence in lowercase):

```
mhhhhhhssgvdlgtenlyfqsMEVFQNHF
EPGVYVCAKCGYELFSSRSKYAHSSPWPAF
TETIHADSVAKRPEHNRSEALKVSCGKCGN
GLGHEFLNDGPKPGQSRFSIFSSSLKFVPK
GKETSA
```

Growth medium, induction protocol: 10ul of a glycerol stock was inoculated into 5ml of TB medium (supplemented with 50µg/ml Kanamycin, 34µg/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 1.6.

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.

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/Amersham)
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 25 mgs of protein was cleaved with 900 ug of TEV protease at 4 degree for 48 hours.
TEV clean up: The TEV cleaved protein was applied to a 1 ml Ni-Sepharose column, already equilibrated with gel filtration buffer (10 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP). The flow through form the column was collected. The eluate from the column was monitored by SDS gel analysis.
Protein concentration: The target protein was concentrated to 10.2 mg/ml using Vivaspin 3K 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 50 nl well solution was equilibrated against well solution containing 2.4M Na(malonate) pH: 7.0. Crystals were mounted in the presence of 30% (v/v) glycerol and flash-cooled in liquid nitrogen.
Data Collection: Resolution: 1.8 Å **, X-ray source:** FRE superbright, single wavelength.