Entry Clone Source: MGC

Entry Clone Accession: IMAGE:3914986

SGC Construct ID: HBP1A-c003

GenBank GI number: gi|21361411

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

Amplified construct sequence:

```
CATATGCACCATCATCATCATCATTC
TTCTGGTGTAGATCTGGGTACCGAGA
ACCTGTACTTCCAATCCATGTCCTGG
CCTTCAACTGTCTGGCACTGTTTTTT
GAAAGGCACACGACTGTGCTTTCATA
AGGGAAGCAATAAGGAATGGCAAGAT
GTTGAAGATTTTGCTAGAGCTGAAGG
CTGTGATAATGAGGAAGATCTTCAAA
TGGGCATTCACAAGGGCTATGGTTCT
GATGGTCTAAAGTTGTTATCACATGA
AGAAAGTGTATCATTTGGCGAGTCTG
TACTGAAGTTGACTTTTGATCCTGGT
ACAGTAGAAGATGGTTTACTTACCGT
AGAGTGTAAGCTGGACCACCCTTTCT
ATGTTAAAAATAAAGGTTGGTCATCA
TTTTATCCAAGCTTGACTGTGGTACA
GCATGGCATTCCATGTTGTGAAGTTC
ATATTGGCGATGTATGTCTACCTCCT
GGACACCCCGATGCCTGACAGTAAAG
GTGGATACGGATCCGAA
```

Final protein sequence (Tag sequence in lowercase):

```
mhhhhhhssgvdlgtenlyfq^smSW
PSTVWHCFLKGTRLCFHKGSNKEWQD
VEDFARAEGCDNEEDLQMGIHKGYGS
DGLKLLSHEESVSFGESVLKLTFDPG
TVEDGLLTVECKLDHPFYVKNKGWSS
FYPSLTVVQHGIPCCEVHIGDVCLPP
GHPDA
```

^ TEV cleavage site

Tags and additions: Cleavable N-terminal His_6 tag.

Host: BL21 (DE3)R3-pRARE2 (Phage resistant strain).

Growth medium, induction protocol: The construct DNA was transformed into competent cells of the expression strain by a standard heat shock procedure. One colony from the transformation was used to inoculate 1ml of TB media containing 50µg/ml kanamycin and 34µg/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day glycerol stocks were prepared from this overnight culture. A glycerol stock of the host expression strain was used to inoculate 50ml of TB media containing 50µg/ml kanamycin and 34µg/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day this

starter culture was used to inoculate 3L of TB media (7.5ml starter culutre used per 1L) containing 50µg/ml kanamycin. When the OD_{600} reached approximately 0.8 the temperature was reduced to 18°C and after a further 30 minutes the cells were induced by the addition of 0.1 mM IPTG. The expression was continued overnight. Cells were harvested by centrifugation at 6000g after which the supernatant was poured out and the cell pellet either placed in a -20°C freezer or used directly for purification.
Lysis buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 10 mM Imidazole; 5% Glycerol; 0.5 mM TCEP; 1 tablet per 50ml protease inhibitor cocktail EDTA-free (Roche).
Extraction buffer, extraction method: Cell pellets were dissolved in apporximately 100ml lysis buffer and broken by passing through a high pressure homogenizer at 15,000psi for 4 cycles. The cell debris was pelleted at 35,000g and the supernatant used for further purification.

Column 1: Ni-sepharose, HisTrap FF, 5ml (GE healthcare).

Column 1 Buffers:
Binding buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 20 mM Imidazole; 0.5 mM TCEP; 1 mM PMSF.
Wash buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 40 mM Imidazole; 0.5 mM TCEP; 1 mM PMSF.
Elution buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 250 mM Imidazole; 0.5 mM TCEP.

Column 1 Procedure: The cell extract was loaded onto 3x5ml Ni-sepharose columns at 5ml/minute on an ÄKTA express system (GE healthcare). The columns were then washed with 20 volumes of lysis buffer, 10 volumes of wash buffer, and then eluted with 5 column volumes elution buffer at 5ml/min. The eluted peak of A_{280} was automatically collected into capillary loops

Column 2: HiLoad 16/60 Superdex, S75 Gel Filtration

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

Column 2 Procedure: The eluted fractions from column 1 were automatically loaded on the gel filtration column pre-equilibrated in GF buffer at 1.2ml/min. Eluted proteins were collected in 1.8ml fractions and analysed on 4-12% SDS-PAGE.

Enzymatic treatment: Fractions containing protein were pooled and incubated with TEV protease overnight at 4°C, at a 1:30 mass ratio of enzyme to protein. The following day TEV-treated sample was passed through a column containing 0.5ml Ni-NTA pre-equilibrated with GF buffer. The column was then washed with 1m of GF Buffer. The flow-through and wash fractions were pooled.

Mass spectrometry characterization: After TEV protease digestion:
Measured mass: 15469.9Da
Expected mass: 15469.5Da

Protein concentration: Protein was concentrated to 18.7mg/ml using an Amicon Ultra-15 centrifugal device (MWCO 10kDa), and frozen at -80°C.

Crystallisation: Crystals were grown by vapour diffusion in sitting drop at 4°C. A sitting drop consisting of 100nl protein (18.7mg/ml) and 50nl well solution (0.3 M ammonium sulfate, 25% w/v PEG3350, 0.1 M Bis-Tris pH 5.5) was equilibrated against 20µl well solution. Crystals were mounted in the presence of 22% (v/v) ethylene glycol and flash-cooled in liquid nitrogen.

Data collection:
Resolution: 2.04Å
X-ray source: Diamond Light Source beamline IO2.