Entry Clone Source: MGC

Entry Clone Accession: IMAGE:5541168

SGC Construct ID: LNX2A-c033

GenBank GI number: gi|24025688

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

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

Mutation: The coding DNA sequence shows a mutation, F338L (5th residue in the final protein sequence for this construct).

Amplified DNA sequence:

```
ATGCACCATCATCATCATCATTCTTCTGG
TGTAGATCTGGGTACCGAGAACCTGTACT
TCCAATCCATGGAGATTCTCCAAGTGGCT
CTTCATAAACGGGACTCTGGTGAACAGCT
TGGCATTAAATTGGTGCGAAGGACAGATG
AGCCAGGGGTTTTTATTCTTGACCTGTTG
GAAGGGGGGTTGGCTGCCCAGGACGGCAG
GCTAAGCAGCAATGACCGAGTGCTGGCCA
TCAATGGGCACGACCTGAAGTATGGAACT
CCGGAGCTTGCTGCCCAGATTATTCAGGC
CAGTGGAGAGAGAGTGAATTTAACAATTG
CTAGACCAGGGAAACCCGAAATCGAACTG
TGA
```

Final protein sequence (Glu336 - Pro424):

sMEILQVALHKRDSGEQLGIKLVRRTDEP
GVFILDLLEGGLAAQDGRLSSNDRVLAIN
GHDLKYGTPELAAQIIQASGERVNLTIAR
PGKP**EIEL**

The N-terminal residues, **sM**, derive from the vector, following TEV protease digestion to remove the N-terminal hexahistidine tag.
The C-terminal residues, **EIEL,** are an addition to promote crystallisation.

Host: 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 preparation: 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: A glycerol stock was used to inoculate 20 ml of TB media containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol, which was placed in a 37°C shaker overnight. The next day this starter culture was used to inoculate 2x 1L of TB media (10 ml starter culture into each) containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol. When the OD_{600} was approximately 1.4, the temperature was reduced to 18°C and the cells were induced by the addition of 0.5 mM IPTG. The expression was continued overnight.

Cell harvest: Cells were spun at 4000x g for 10 mins. The cells were resuspended in 50 ml of Binding Buffer and placed in a -80°C freezer.

Binding Buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 5 mM Imidazole pH 7.5, 0.5 mM TCEP.
Purification: Cell Lysis: The resuspended cell pellet was lysed by sonication. The cell debris was spun down (17000 rpm, JA17 rotor, 60 min).
Binding Buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 5 mM Imidazole pH 7.5, 0.5 mM TCEP.
Column 1: Ni-NTA (5 ml volume in a gravity-flow column).
Column 1 Buffers: Binding Buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 5 mM Imidazole pH 7.5, 0.5 mM TCEP; **Wash Buffer:** 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 40 mM Imidazole pH 7.5, 0.5 mM TCEP; **Elution Buffer:** 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole pH 7.5, 0.5 mM TCEP.
Column 1 Procedure: The clarified cell extract was passed through the column. The column was then washed with 2x 20 ml of Wash Buffer. The protein was eluted with 10 ml of Elution Buffer.
Column 2: S75 16/60 Gel Filtration.
Column 2 Buffers: GF Buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 0.5 mM TCEP.
Column 2 Procedure: The elution buffer fraction from column 1 was concentrated to 2 ml volume. The concentrated sample was injected onto an S200 16/60 column pre-equilibrated in GF Buffer.
TEV protease digestion: TEV protease was added to the pooled fractions from the gel filtration. The sample was left at 4°C overnight. The sample was then passed through 0.5 ml of Ni-NTA resin.
Concentration: The sample was concentrated to 5.8 mg/ml (measured by 280 nm absorbance).
Mass spectrometry characterisation: Measured: 10296.7; **Expected:** 10297.6 (with F338L mutation)
Crystallisation: Crystals grew from a 2:1 ratio of protein to precipitant solution (40% PEG300, 0.1M citrate/PO_4 pH 4.2), using the vapour diffusion method. Crystals were grown in 150 nL drops at 20°C
Data Collection: Crystals were cryo-protected by equilibration into precipitant solution containing 40% PEG300, and then flash frozen in liquid nitrogen. **Resolution:** 1.3 Å. Data was collected at the Swiss Light Source, beamline X10.