Entry Clone Source: MGC

Entry Clone Accession: IMAGE:4867373

SGC Construct ID: RPS6KA1A-c036

GenBank GI number: gi|20149547

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

Coding DNA sequence:

```
ATGCACCATCATCATCATCATTCTTCTGGT
GTAGATCTGGGTACCGAGAACCTGTACTTC
CAATCCATGGTTTTTAGTGACGGCTACGTG
GTAAAGGAGACAATTGGTGTGGGCTCCTAC
TCTGAGTGCAAGCGCTGTGTCCACAAGGCC
ACCAACATGGAGTATGCTGTCAAGGTCATT
GATAAGAGCAAGCGGGATCCTTCAGAAGAG
ATTGAGATTCTTCTGCGGTATGGCCAGCAC
CCCAACATCATCACTCTGAAAGATGTGTAT
GATGATGGCAAACACGTGTACCTGGTGACA
GAGCTGATGCGGGGTGGGGAGCTGCTGGAC
AAGATCCTGCGGCAGAAGTTCTTCTCAGAG
CGGGAGGCCAGCTTTGTCCTGCACACCATT
GGCAAAACTGTGGAGTATCTGCACTCACAG
GGGGTTGTGCACAGGGACCTGAAGCCCAGC
AACATCCTGTATGTGGACGAGTCCGGGAAT
CCCGAGTGCCTGCGCATCTGTGACTTTGGT
TTTGCCAAACAGCTGCGGGCTGAGAATGGG
CTCCTCATGACACCTTGCTACACAGCCAAC
TTTGTGGCGCCTGAGGTGCTGAAGCGCCAG
GGCTACGATGAAGGCTGCGACATCTGGAGC
CTGGGCATTCTGCTGTACACCATGCTGGCA
GGATATACTCCATTTGCCAACGGTCCCAGT
GACACACCAGAGGAAATCCTAACCCGGATC
GGCAGTGGGAAGTTTACCCTCAGTGGGGGA
AATTGGAACACAGTTTCAGAGACAGCCAAG
GACCTGGTGTCCAAGATGCTACACGTGGAT
CCCCACCAGCGCCTCACAGCTAAGCAGGTT
CTGCAGCATCCATGGGTCACCCAGAAAGAC
AAGCTTCCCCAAAGCCAGCTGTCCCACCAG
GACCTACAGCTTGTGAAGGGAGCCATGGCT
GCCACGTACTCCGCACTCAACAGCTCCAAG
CCCACCCCCCAGCTGAAGCCCATCGAGTCA
TCCATCCTGGCCCAGCGGCGAGTGAGGAAG
TTGCCATCCACCACCCTGTGA
```

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

Expressed sequence (tag sequence in lowercase):

```
mhhhhhhssgvdlgtenlyfqsmVFSDGYV
VKETIGVGSYSECKRCVHKATNMEYAVKVI
DKSKRDPSEEIEILLRYGQHPNIITLKDVY
DDGKHVYLVTELMRGGELLDKILRQKFFSE
REASFVLHTIGKTVEYLHSQGVVHRDLKPS
NILYVDESGNPECLRICDFGFAKQLRAENG
LLMTPCYTANFVAPEVLKRQGYDEGCDIWS
LGILLYTMLAGYTPFANGPSDTPEEILTRI
GSGKFTLSGGNWNTVSETAKDLVSKMLHVD
```

PHQRLTAKQVLQHPWVTQKDKLPQSQLSHQ
DLQLVKGAMAATYSALNSSKPTPQLKPIES
SILAQRRVRKLPSTTL
(Val413 to Leu735)
The N-terminal residues, mhhhhhhssgvdlgtenlyfqsm, derive from the vector.

Expression strain:BL-21(DE3)-R3-Rosetta (A homemade phage resistant version of BL21(DE3) containing the pRARE2 plasmid from Rosetta II (DE3) cells).

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 50 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 this starter culture was used to inoculate 3x 1L of TB media (13 ml starter culture into each) containing 35 µg/ml kanamycin. After 4 hours, when the OD_{600} was 1.0, the temperature was reduced to 19°C. After a further 45 minutes the cells were induced by the addition of 0.75 mM IPTG. The expression was continued overnight.

Cell harvest: Cells were spun at 6238x g for 10 mins at 4°C. The cells were resuspended in 105 ml of Lysis Buffer with the addition of a 1:5000 dilution of Calbiochem Protease inhibitor set VII. The resuspended cell pellet was placed in a -80°C freezer.

Cell Lysis: The resuspended cell pellet was lysed by sonication. PEI (polyethyleneimine) was added to a final concentration of 0.15 % and the cell debris and precipitated DNA were spun down (17000 rpm, JA17 rotor, 45 min).

Lysis Buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 5 mM Imidazole pH 7.4, 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 7.4, 500 mM NaCl, 5% Glycerol, 5 mM Imidazole pH 7.4, 0.5 mM TCEP; **Wash Buffer:** 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 25 mM Imidazole pH 7.4, 0.5 mM TCEP; **Elution Buffer:** 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole pH 7.4, 0.5 mM TCEP

Column 1 Procedure: The clarified cell extract was passed through the column. The column was then washed with Binding Buffer (100 ml) and Wash Buffer (50 ml). The protein was eluted with 25 ml of Elution Buffer.

Column 2: S200 16/60 Gel Filtration.

Column 2 Buffers: GF Buffer: 50 mM Hepes pH 7.4, 300 mM NaCl, 1 mM DTT

Column 2 Procedure: The elution buffer fraction from column 1 was concentrated to 4 ml volume in a 30 kDa MW cutoff spin concentrator. The concentrated sample was injected onto an S200 16/60 column pre-equilibrated in GF Buffer at 1.0 ml/min. 1.75 ml fractions were collected.

Concentration: The pooled fractions from Column 2 were concentrated to 21 mg/ml (measured by 280 nm absorbance), distributed into aliquots and frozen at -80°C.

Mass spectrometry characterization :
Measured: 38929.2
Expected: 38927.1

Crystallization: Crystals grew from a 1:2 ratio of protein to precipitant solution (20% PEG3350, 0.2M sodium formate), using the vapour diffusion method.

Data Collection: Resolution: 2.3 Å. Crystals were cryo-protected by equilibration into precipitant solution containing 25% Ethylene glycol, and then flash frozen in liquid nitrogen. Data was collected at the Swiss Light Source, beamline X10.