Entry Clone Source: MGC

Entry Clone Accession: IMAGE:3357140

SGC Construct ID: ENOSF1A-c001

GenBank GI number: gi|3454185

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

Amplified construct sequence:

CATATGCACCATCATCATCATCATTC
TTCTGGTGTAGATCTGGGTACCGAGA
ACCTGTACTTCCAATCCATGGTGCGC
GGCAGGATCTCCCGGCTCTCGGTCCG
GGACGTGCGCTTCCCCACGTCGCTTG
GGGGCCACGGCGCGGACGCCATGCAC
ACGGACCCTGACTACTCGGCTGCCTA
TGTCGTCATAGAAACTGATGCAGAAG
ATGGAATCAAGGGGTGTGGAATTACC
TTCACTCTGGGAAAAGGCACTGAAGT
TGTTGTCTGTGCTGTGAATGCCCTCG
CCCACCATGTGCTCAACAAGGACCTC
AAGGACATTGTTGGTGACTTCAGAGG
CTTCTATAGGCAGCTCACAAGTGATG
GGCAGCTCAGATGGATTGGTCCAGAA
AAGGGCGTGGTGCACCTGGCGACAGC
GGCCGTCCTAAACGCGGTGTGGGACT
TGTGGGCCAAGCAGGAGGGAAAGCCT
GTCTGGAAGTTACTTGTGGACATGGA
TCCCAGGATGCTGGTATCCTGCATAG
ATTTCAGGTACATCACTGATGTCCTG
ACTGAGGAGGATGCCCTAGAAATACT
GCAGAAAGGTCAAATTGGTAAAAAAG
AAAGAGAGAAGCAAATGCTGGCACAA
GGATACCCTGCTTACACGACATCGTG
CGCCTGGCTGGGGTACTCAGATGACA
CGTTGAAGCAGCTCTGTGCCCAGGCG
CTGAAGGATGGCTGGACCAGGTTTAA
AGTAAAGGTGGGTGCTGATCTCCAGG
ATGACATGCGAAGATGCCAAATCATC
CGAGACATGATTGGACCGGAAAAGAC
TTTGATGATGGATGCCAACCAGCGCT
GGGATGTGCCTGAGGCGGTGGAGTGG
ATGTCCAAGCTGGCCAAGTTCAAGCC
ATTGTGGATTGAGGAGCCAACCTCCC
CTGATGACATTCTGGGGCACGCCACC
ATTTCCAAGGCACTGGTCCCATTAGG
AATTGGCATTGCCACAGGAGAACAGT
GCCACAATAGAGTGATATTTAAGCAA
CTCCTACAGGCGAAGGCCCTGCAGTT
CCTCCAGATTGACAGTTGCAGACTGG
GCAGTGTCAATGAGAACCTCTCAGTA
TTGCTGATGGCCAAAAAGTTTGAAAT

TCCTGTTTGCCCCCATGCTGGTGGAG
TTGGCCTCTGTGAACTGGTGCAGCAC
CTGATTATATTTGACTACATATCAGT
TTCTGCAAGCCTTGAAAATAGGGTGT
GTGAGTATGTTGACCACCTGCATGAG
CATTTCAAGTATCCCGTGATGATCCA
GCGGGCTTCCTACATGCCTCCCAAGG
ATCCCGGCTACTCAACAGAAATGAAG
GAGGAATCTGTAAAGAAACACCAGTA
TCCAGATGGTGAAGTTTGGAAGAAAC
TCCTTCCTGCTTGACAGTAAAGGTGG
ATACGGATCCGAA

Final protein sequence (Tag sequence in lowercase):
mhhhhhhssgvdlgtenlyfq^smVR
GRISRLSVRDVRFPTSLGGHGADAMH
TDPDYSAAYVVIETDAEDGIKGCGIT
FTLGKGTEVVVCAVNALAHHVLNKDL
KDIVGDFRGFYRQLTSDGQLRWIGPE
KGVVHLATAAVLNAVWDLWAKQEGKP
VWKLLVDMDPRMLVSCIDFRYITDVL
TEEDALEILQKGQIGKKEREKQMLAQ
GYPAYTTSCAWLGYSDDTLKQLCAQA
LKDGWTRFKVKVGADLQDDMRRCQII
RDMIGPEKTLMMDANQRWDVPEAVEW
MSKLAKFKPLWIEEPTSPDDILGHAT
ISKALVPLGIGIATGEQCHNRVIFKQ
LLQAKALQFLQIDSCRLGSVNENLSV
LLMAKKFEIPVCPHAGGVGLCELVQH
LIIFDYISVSASLENRVCEYVDHLHE
HFKYPVMIQRASYMPPKDPGYSTEMK
EESVKKHQYPDGEVWKKLLPA

^ TEV cleavage site

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

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

Transformation: The construct DNA was transformed into competent cells of the expression strain by a standard heat shock procedure.

Glycerol stock preparation: One colony from the transformation was used to inoculate 1 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 glycerol stocks were prepared from this overnight culture.

Expression: A glycerol stock was used to inoculate 50 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 12L of TB media (3.5 ml starter culture used per 1L) containing 50 µg/ml kanamycin. When the OD_{600} reached approximately 1.0 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

Cell harvest: Cells were harvested by centrifugation at 6000 x g after which the supernatant was poured out and the cell pellet either placed in a -20°C freezer or used directly for purification.

Cell Lysis: Cell pellets were dissolved in approximately 150ml lysis buffer and broken by homogenization by 5 passes at 12,000 psi. The cell debris was pelleted at 35,000 x g and the supernatant used for further purification
Lysis buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 10 mM Imidazole pH 7.4, 0.5 mM TCEP, 1 tablet per 50 ml protease inhibitor cocktail EDTA-free (Roche)
Extraction buffer, extraction method: Frozen pellets were thawed and supplemented with TCEP, Benzonase and protease inhibitors. Cells were lysed by sonication. Nucleic acids and cell debris were removed by adding 0.15% PEI (polyethyleneimine), then centrifuged for further 30 minutes at 17,000rpm.

Column 1: Ni-NTA (2.5 ml volume in a gravity-flow column).

Column 1 Buffers:
Binding buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 10 mM Imidazole pH 7.4, 0.5 mM TCEP
Wash buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 40 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 incubated with 2.5 ml of Ni-NTA pre-equilibrated with lysis buffer for 1 hour at 4°C with rotation after which it was passed through a glass column. The column was then washed with Binding Buffer (60 ml) and Wash Buffer (50 ml). The protein was eluted with 25 ml of Elution Buffer in 5 x 5 ml fractions.

Column 2: Gel filtration, HiLoad 16/60 Superdex S75 prep grade, 120ml (GE Healthcare).

Column 2 GF Buffer: 10 mM Hepes pH 7.4, 500 mM NaCl, 0.5 mM TCEP, 5% Glycerol

Column 2 Procedure: The wash buffer fractions and elution buffer fractions from column 1 were pooled separately and concentrated to 5 ml with a 30 kDa mwco spin concentrator and injected onto an s200 16/60 column (pre-equilibrated in GF Buffer) at 1.0 ml/min. 1.0 ml fractions were collected.
The protein eluted at between 80 ml and 90 ml volume.

Column 3: TEV cleavage/ Ni-NTA rebind.

Column 3 Wash Buffer: 10 mM Hepes pH 7.4, 500 mM NaCl, 0.5 mM TCEP, 5% Glycerol

Column 3 Procedure: Protein from fractions eluted at 80-90 ml from s200 gel filtration were pooled and incubated with 1:20 mol:mol TEV protease overnight at 4°C. Then protein plus TEV was passed through a column containing 0.5 ml Ni-NTA pre-equilibrated with GF Buffer. Column was washed 2ml of GF Buffer and 2ml Elution Buffer. Protein was poorly cut and the majority of protein was found in the Elution buffer fraction..

Column 4: 1ml Resource Q Cation Exchange

Column 4 Buffer A: 50 mM Tris pH 8.5, 50 mM NaCl

Column 4 Buffer B: 50 mM Tris pH 8.5, 2 M NaCl

Column 4 Procedure: Protein from Ni-rebind elution, approximately 4 ml, was diluted to 40 ml using Buffer A and injected into a 1ml Resource Q column. Protein was eluted using a linear gradient of 0-25% Buffer B over 35 column volumes at 1ml/min. 1.0 ml fractions were collected.

Concentration: The fraction that was found in the flow-through was concentrated to 35 mg/ml using a 30 kDa mwco concentrator

Mass spectrometry characterization:
Observed mass: 49505.0 Da
Expected mass: 49502.4 Da

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

Data collection:
Resolution: 2.00Å.
X-ray source: Diamond Light Source beamline I03.