Entry Clone Source: Synthetic

Entry Clone Accession: n/a

SGC Construct ID: MAST4A-c010

GenBank GI number: gi|51464991

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

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

Final protein sequence:

smQPIVIHSSGKNYGFTIRAIRVYVGDSD
IYTVHHIVWNVEEGSPACQAGLKAGDLIT
HINGEPVHGLVHTEVIELLLKSGNKVSIT
TTPFENTETSV

The N-terminal 2 residues, **sm**, derive from the vector, following TEV protease cleavage of the hexahistidine tag.

Coding DNA sequence:

ATGCACCATCATCATCATCATTCTTCTGG
TGTAGATCTGGGTACCGAGAACCTGTACT
TCCAATCCATGCAGCCGATCGTGATTCAT
AGCAGCGGCAAAAATTATGGCTTTACCAT
TCGCGCGATCCGTGTGTATGTGGGCGATA
GCGATATTTATACCGTGCATCATATTGTG
TGGAATGTGGAAGAAGGCAGCCCGGCGTG
CCAGGCGGGCCTGAAAGCCGGCGATCTGA
TTACCCATATTAACGGTGAACCGGTTCAT
GGCCTGGTGCATACCGAAGTGATTGAACT
GCTGCTGAAAAGCGGCAACAAAGTGAGCA
TTACCACCACCCCGTTTGAAAATACCGAA
ACCAGCGTT

Expression strain: 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 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 2x 1L of TB media (18 ml starter culture into each) containing 50 µg/ml kanamycin. After 3 hours the temperature was reduced to 20°C. After a further 30 minutes the cells were induced by the addition of 0.5 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 60 ml of Lysis Buffer with the addition of 0.2 mM PMSF. The resuspended cell pellet was placed in a -80°C freezer.

Lysis Buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 5 mM Imidazole pH 7.4, 0.5 mM TCEP

Cell Lysis: The resuspended cell pellet was lysed by sonication. PEI (polyethyleneimine) was added to a final concentration of 0.25 % and the cell debris and precipitated DNA were spun down (18000 rpm, JA18 rotor, 90 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 (2 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 (25 ml) and Wash Buffer (25 ml). The protein was eluted with 10 ml of Elution Buffer.
Column 2: S200 16/60 Gel Filtration.
Column 2 Buffers: GF Buffer: 25 mM Hepes pH 7.4, 150 mM NaCl, 0.5 mM TCEP.
Column 2 Procedure: The wash buffer and elution buffer fractions from column 1 were combined and concentrated to 4 ml volume in a 3 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.
TEV protease digestion: Gel filtration fractions containing MAST4A PDZ domain were combined. TEV protease was added and the sample left at 4°C overnight.
Rebinding of impurities to Ni-NTA: The protein was passed through a gravity-flow column containing Ni-NTA resin (2 ml resin volume, pre-equilibrated into GF Buffer).
Concentration: The TEV protease cleaved protein was concentrated to 16.7 mg/ml (measured by 280 nm absorbance), distributed into aliquots and frozen at -80°C.
Mass spectrometry characterisation: Measured: 10640.6; **Expected:** 10640.
Crystallisation: Crystals grew from a 2:1 ratio of protein to precipitant solution (0.75M NaH_2PO_4, 0.91M K_2HPO_4), using the vapour diffusion method.
Data Collection: 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.