Entry Clone Source: MGC

Entry Clone Accession: IMAGE:2957960

SGC Construct ID: PTK9LA-c037

GenBank GI number: gi|6005846

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

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

Final protein sequence:
smPLQPEAQRALQQLKQKMVNYIQMKLDL
ERETIELVHTEPTDVAQLPSRVPRDAARY
HFFLYKHTHEGDPLESVVFIYSMPGYKCS
IKERMLYSSCKSRLLDSVEQDFHLEIAKK
IEIGDGAELTAEFL**D**DEVH

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

Residue **D**309 represents a difference from the native sequence, caused by a cloning artifact.

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 prepataion: A number of colonies from the transformation were used to innoculate 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: 50 ml of a glycerol stock was used to innoculate 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 innoculate 2x 1L of TB media (18 ml starter culture into each) containing 50 µg/ml kanamycin. After 2 hours the temperature was reduced to 20°C. After a further 1.5 hours 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. Each 1L cell pellet was resuspended in 30 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 4 passes through an Emulsiflex C5 homogeniser, collecting a final volume of approximately 150 ml. 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, 70 min).

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

Purification: The protein was purified using an AktaExpress system to do automated Ni2+-affinity followed by gel filtration.

Column 1: HisTrap 1 ml.

Column 1 Procedure: The clarified cell extract was passed through the column at a flow rate of 0.8 ml/min. The column was then washed Binding Buffer until a stable UV baseline was achieved. The

column was then washed with Wash Buffer until a stable UV baseline was achieved. The protein was eluted with 5 ml of Elution Buffer.

Column 2: S200 16/60.

Column 2 Procedure: The desalting column was pre-equilibrated with GF Buffer. The HisTrap eluant was loaded on the GF column automatically after the HisTrap elution at a flow rate of 1.2 ml/min. Eluted proteins were collected in 1.8 ml fractions. The fractions containing protein were identified on a coomasie blue stained gel.

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

GF Buffer: 25 mM Hepes pH 7.4, 150 mM NaCl, 0.5 mM TCEP

TEV protease digestion: The fractions from gel filtration containing PTK9LA were pooled and 40 ml of TEV protease (about 2 mg/ml) was added. The digestion was left overnight at 4 °C. After 24 hours the TEV protease digestion had not proceeded to completion so an additional 400 ml of TEV protease solution was added and the digestion left for an additional 24 hours.

Rebinding of impurities to Ni-NTA: The protein was passed in series through two gravity-flow columns, each containing 1 ml of Ni-NTA resin pre-equilibrated into GF Buffer.

Concentration: The TEV protease cleaved PTK9LA 2nd ADF-H domain was concentrated to 5.8 mg/ml (measured by 280 nm absorbance), distributed into aliquots and frozen at -80°C.

Mass spec. characterisation: Measured: 15695.6; Expected: 15743.9. The mass discrepancy of -48.3 corresponds to a Tyr to Asp mutation at residue 309, which was confirmed by DNA sequencing of the PTK9LA-c037 construct.

Crystallisation: Crystals grew from a 1:1 ratio of protein to precipitant solution (2.6 M Na Malonate pH 7.0, 0.1 M BisTris pH 6.5) using the vapour diffusion method. The drop size was 270 nL, and the reservoir volume was 40 mL.

Data Collection: Crystals were cryo-protected by equilibration into 3.4 M Na Malonate pH 7.0, and then flash frozen in liquid nitrogen. Data was collected at the Swiss Light Source, beamline X10. **Resolution:** 1.8 Å.