Entry Clone Source: MGC

Entry Clone Accession: IMAGE:4747045

SGC Construct ID: TGM1A-c116

GenBank GI number: gi|4507475

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

Amplified construct sequence:
CATATGCACCATCATCATCATCATTC
TTCTGGTGTAGATCTGGGTACCGAGA
ACCTGTACTTCCAATCCATGCTCTCC
CTCACGTTACTGGGAGCAGCAGTGGT
TGGCCAGGAGTGTGAAGTACAGATTG
TCTTCAAGAACCCCCTTCCCGTCACC
CTCACCAATGTCGTCTTCCGGCTCGA
AGGCTCTGGGTTACAGAGGCCCAAGA
TCCTCAACGTTGGGGACATTGGAGGC
AATGAAACAGTGACACTGCGCCAGTC
GTTTGTGCCTGTGCGACCAGGCCCCC
GCCAGCTCATTGCCAGCTTGGACAGC
CCACAGCTCTCCCAGGTGCACGGTGT
CATCCAGGTGGATGTGGCCTAACAGT
AAAGGTGGATACGGATCCGAA

Final protein sequence (Tag sequence in lowercase):
mhhhhhhssgvdlgtenlyfq^smLS
LTLLGAAVVGQECEVQIVFKNPLPVT
LTNVVFRLEGSGLQRPKILNVGDIGG
NETVTLRQSFVPVRPGPRQLIASLDS
PQLSQVHGVIQVDVA

^ TEV cleave site

Tags and additions: N-terminal TEV cleavable His6 tag.

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

Growth medium, induction protocol: 10µl of glycerol stock of host strain BL21(DE3)-R3-pRARE2 was used to inoculate 100ml of TB (Terrific Broth) supplemented with 50µg/ml kanamycin. This starter culture was grown overnight at 37°C and used next day to inoculate 8L TB (5ml starter culture per 1 litre) containing 50µg/ml kanamycin. The culture was grown at 37°C until the OD_{600} reached ~1. After that the temperature was lowered to 18° and protein production was induced by addition of 0.1 mM IPTG. The expression was continued overnight at that temperature. The next day cells were harvested by centrifugation at 4000 rpm for 20 minutes at 4°C then the supernatant was discarded and pellets resuspended in binding buffer and stored at -80°C.
Lysis buffer: 50 mM HEPES pH 7.5; 500 mM NaCl; 5% Glycerol; 10 mM Imidazole, pH 7.5; 0.5 mM TCEP; 1 mM PMSF.
Extraction buffer, extraction method: Frozen cells, previously resuspended, were thawed, and supplemented with benzonase (25U/ml, 2µl of benzonase per 50ml of buffer). Cells were

passed 4 times through an Emulsiflex C5 high-pressure homogeniser, collected and centrifuged for 60 min at 15500 rpm (Beckman JLA 16.25).

Column 1: Ni-sepharose, HisTrap FF, 5ml (GE healthcare).

Column 1 Buffer:
Wash Buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 40 mM Imidazole; 5% glycerol; 1 mM PMSF; 0.5 mM TCEP.
Elution Buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% Glycerol; 250 mM Imidazole; 0.5 mM TCEP.

Column 1 Procedure: The cell extract was loaded onto 2x5ml Ni-sepharose columns at 4ml/minute on an ÄKTA Express system (GE healthcare). The columns were then washed with 20 column volumes of binding buffer, 10 column volumes of wash buffer, and then eluted with 5 column volumes of elution buffer at 5ml/min. The eluted peak of A_{280} was autmatically collectd.

Column 2: Gel filtration. HisLoad S200 16/60.

Column 2 Buffers: 10 mM HEPES, pH 7.5; 500 mM NaCl; 5% glycerol; 0.5 mM TCEP.

Column 2 Procedure: The eluted fractions from Ni-sepharose were automatically loaded on the gell filtration column pre-equilibrated in GF buffer at 1.2ml/min. Eluted proteins were collected in 1.8ml fractions and analysed by SDS-PAGE.

Enzymatic treatment: Fractions containing TGM1A were pooled and 1mg of TEV protease was added per 45mg protein. The digestion was performed overnight at 4°C. The following day protein sample was loaded onto Ni-sepharose column (1ml slurry) pre-equilibrated with GF buffer to remove uncleaved protein. The flow-through and wash fractions were pooled and concentrated using Amicon Ultra-15 concentrators with 10kDa cutoff.

Protein concentration: Protein was concentrated to 12mg/ml and frozen at -80°C.

Mass spectrometry characterization:
Measured: 12793.7 Da (ESI-MS).
Expected: 12793.7 Da
Mass spec analysis reveals that the purified fractions contain both tag-cleaved and uncleaved protein.

Crystallisation: Crystals were grown at 20°C by vapour diffusion in sitting drops mixing protein (12mg/ml) and well solution containing 0.7M ammonium sulphate, 1% (v/v) PEG3350, 0.1 M BTS-TRIS pH 5.5 at a protein to precipitant ratio of 2:1. Crystals were cryo-protected using 25% (v/v) ethylene glycol supplemented to the well solution and flash cooled in liquid nitrogen.

Data Collection:
Resolution: 2.3 Å
X-ray source: Diamond Light Source beamline I04.