TSTA3A (4B8W) Materials & Methods

Entry clone source: MGC

SGC Construct ID: TSTA3A-c013

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

DNA sequence:

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ATGGGTGAACCCCAGGGATCCATGCG
GATTCTAGTGACAGGGGGCTCTGGGC
TGGTAGGCAAAGCCATCCAGAAGGTG
GTAGCAGATGGAGCTGGACTTCCTGG
AGAGGACTGGGTGTTTGTCTCCTCTA
AAGACGCCGATCTCACGGATACAGCA
CAGACCCGCGCCCTGTTTGAGAAGGT
CCAACCCACACACGTCATCCATCTTG
CTGCAATGGTGGGGGGCCTGTTCCGG
AATATCAAATACAATTTGGACTTCTG
GAGGAAAAACGTGCACATGAACGACA
ACGTCCTGCACTCGGCCTTTGAGGTG
GGCGCCCGCAAGGTGGTGTCCTGCCT
GTCCACCTGTATCTTCCCTGACAAGA
CGACCTACCCGATAGATGAGACCATG
ATCCACAATGGGCCTCCCCACAACAG
CAATTTTGGGTACTCGTATGCCAAGA
GGATGATCGACGTGCAGAACAGGGCC
TACTTCCAGCAGTACGGCTGCACCTT
CACCGCTGTCATCCCCACCAACGTCT
TCGGGCCCCACGACAACTTCAACATC
GAGGATGGCCACGTGCTGCCTGGCCT
CATCCACAAGGTGCACCTGGCCAAGA
GCAGCGGCTCGGCCCTGACGGTGTGG
GGTACAGGGAATCCGCGGAGGCAGTT
CATATACTCGCTGGACCTGGCCCAGC
TCTTTATCTGGGTCCTGCGGGAGTAC
AATGAAGTGGAGCCCATCATCCTCTC
CGTGGGCGAGGAAGATGAGGTCTCCA
TCAAGGAGGCAGCCGAGGCGGTGGTG
GAGGCCATGGACTTCCATGGGGAAGT
CACCTTTGATACAACCAAGTCGGATG
GGCAGTTTAAGAAGACAGCCAGTAAC
AGCAAGCTGAGGACCTACCTGCCCGA
CTTCCGGTTCACACCCTTCAAGCAGG
CGGTGAAGGAGACCTGTGCTTGGTTC
ACTGACAACTACGAGCAGGCCCGGAA
GTGA
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Final protein sequence (His$_6$ affinity Tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfq^smRI
LVTGGSGLVGKAIQKVVADGAGLPGE
DWVFVSSKDADLTDTAQTRALFEKVQ
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PTHVIHLAAMVGGLFRNIKYNLDFWR
KNVHMNDNVLHSAFEVGARKVVSCLS
TCIFPDKTTYPIDETMIHNGPPHNSN
FGYSYAKRMIDVQNRAYFQQYGCTFT
AVIPTNVFGPHDNFNIEDGHVLPGLI
HKVHLAKSSGSALTVWGTGNPRRQFI
YSLDLAQLFIWVLREYNEVEPIILSV
GEEDEVSIKEAAEAVVEAMDFHGEVT
FDTTKSDGQFKKTASNSKLRTYLPDF
RFTPFKQAVKETCAWFTDNYEQARK

^ TEV protease recognition site

Tags and additions: Cleavable N-terminal His6 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 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: 5 µl 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 6L of TB media (1 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 the cells were induced by the addition of 0.2 mM IPTG. The expression was continued overnight at 18°C.

Cell harvest: Cells were harvested by centrifugation at 5000 rpm for 11 min at 4°C after which the supernatant was discarded and the cell pellet was frozen at -20°C until future use.

Cell Lysis: Cell pellets from 6 liter expression were slowly thawed on ice. Afterwards the cell pellets were dissolved in approximately 30-40 ml binding buffer and broken by using an Avestin C-5 homogenizer. The samples were passed through the homogenizer at least three times or until the lysate lost viscosity. After lysis the pellet was separated from the supernatant by centrifugation at 4°C for 45 min at 16,500 rpm. The clear supernatant was transferred to a fresh 50 ml Falcon tube for further purification.
Binding buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 5 mM Imidazole, 0.5 mM TCEP, 1 mM PMSF

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

Column 1 Buffers:
Binding buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 5 mM Imidazole, 0.5 mM TCEP, 1 mM PMSF
Wash buffer: 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 5% Glycerol, 30 mM Imidazole
Elution buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole

Column 1 Procedure: The clarified cell extract was further purified on a 2.5 ml of Ni-NTA column. The supernatant, pre-equilibrated with binding buffer, was applied on the column twice before washing and eluting. During the wash step ten times 5 ml portions of wash buffer were added to the column. The protein was eluted with three times 5 ml of Elution Buffer

Column 2: Superdex 200 10/300 column

Column 2 Buffers:
Gel Filtration buffer: 10 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 1mM PMSF, 0.5mM TCEP

Column 2 Procedure: The eluted fractions from column 1 were pooled separately and concentrated to 5 ml with a 30 kDa mwco spin concentrator. The 5 ml protein sample was injected onto the Superdex 200 column pre-equilibrated with gel filtration buffer, and 1 ml fractions were collected at 1.0 ml/min. The protein eluted between 85 ml and 100 ml column volume.

Concentration: The eluted protein was concentrated and 50 µl aliquots at a concentration of 12 mg/ml were stored at -80°C.

Mass spec characterization:
Expected mass: 37758.7 Da (with tag), Measured mass: 38240.3 Da (with tag and after methylation)

Crystallization: Crystals were grown by vapour diffusion in hanging drop at 20°C by setting up 12 mg/ml of protein in the presence of 5 mM NADP+ and 10 mM GDP. Pyramidal crystals appeared in a hanging drop consisting of 1 µl protein and 1 µl well solution which had been equilibrated against 500 µl well solution containing 25% PEG 3350, 0.1 M bis-tris-propane pH 7.5, 0.3 M NaBr and 10% (v/v) ethylene glycol. Crystals were mounted in the presence of 30% ethylene glycol and flash cooled in liquid nitrogen.

Data Collection: Resolution: 2.70 Å
X-ray source: Swiss Light Source beamline X10