HGSA (4AVX) Materials & Methods

Entry Clone Source: MGC

SGC Construct ID: HGSA-c001

Vector: pNIC28-Bsa4. Details [PDF]; Sequence [FASTA] or [GenBank]. T7/lac regulated, N-terminal His-tag, TEV, LIC cloning using BsaI cleavage/T4 polymerase, SacB stuffer fragment, pET28 backbone

DNA sequence:

```
TACTTCCAATCCATGGGGCGAGGCAG
CGGCACCTTCGAGCGTCTCCTAGACA
AGGCGACCAGCCAGCTCCTGTTGGAG
ACAGATTGGGAGTCCATTTTGCAGAT
CTGCGACCTGATCCGCCAAGGGGACA
CACAAGCAAAATATGCTGTGAATTCC
ATCAAGAAGAAAGTCAACGACAAGAA
CCCACACGTCGCCTTGTATGCCCTGG
AGGTCATGGAATCTGTGGTAAAGAAC
TGTGGCCAGACAGTTCATGATGAGGT
GGCCAACAAGCAGACCATGGAGGAGC
TGAAGGACCTGCTGAAGAGACAAGTG
GAGGTAAACGTCCGTAACAAGATCCT
GTACCTGATCCAGGCCTGGGCGCATG
CCTTCCGGAACGAGCCCAAGTACAAG
GTGGTCCAGGACACCTACCAGATCAT
GAAGGTGGAGGGGCACGTCTTTCCAG
AATTCAAAGAGAGCGATGCCATGTTT
GCTGCCGAGAGAGCCCCAGACTGGGT
GGACGCTGAGGAATGCCACCGCTGCA
GGGTGCAGTTCGGGGTGATGACCCGT
AAGCACCACTGCCGGGCGTGTGGGCA
GATATTCTGTGGAAAGTGTTCTTCCA
AGTACTCCACCATCCCCAAGTTTGGC
ATCGAGAAGGAGGTGCGCGTGTGTGA
GCCCTGCTACGAGCAGCTGAACAGGA
AAGCGGAGGGATGACAGTAAAGGTGG
ATA
```

Final protein sequence (Tag sequence in lowercase):

```
mhhhhhhssgvdlgtenlyfq^sMGR
GSGTFERLLDKATSQLLLETDWESIL
QICDLIRQGDTQAKYAVNSIKKKVND
KNPHVALYALEVMESVVKNCGQTVHD
EVANKQTMEELKDLLKRQVEVNVRNK
ILYLIQAWAHAFRNEPKYKVVQDTYQ
IMKVEGHVFPEFKESDAMFAAERAPD
WVDAEECHRCRVQFGVMTRKHHCRAC
GQIFCGKCSSKYSTIPKFGIEKEVRV
CEPCYEQLNRKAEG
```

^ TEV protease recognition site

Tags and additions: Cleavable N-terminal His6 tag

Host: BL21(DE3)-R3-pRARE2. Phage-resistant derivative of BL21 (DE3), with pRARE2 plasmid encoding rare codon tRNAs (chloramphenicol-resistant).

Growth Medium & Induction Protocol: A glycerol stock was used to inoculate a 50ml starter culture containing LB media with 50µg/ml Kanamycin and 34µg/ml Chloramphenicol. The starter culture was grown overnight at 37°C with shaking at 200 rpm. The following morning, three flasks containing 1L TB/Kanamycin were each inoculated with 10 ml of the starter culture. Cultures were incubated at 37°C with shaking at 180 rpm until an OD_{600}nm = 0.8 was reached. The flasks were then cooled down to 18°C and 1mM IPGT was added to induce protein expression overnight. Cells were harvested by centrifugation at 5000 rpm at 4°C for 15 min. Cell pellets from each flask were resuspended in 15ml Binding buffer (50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole).

Extraction buffer, extraction method: The cells were lysed by ultrasonication over 25 min with the sonicator pulsing ON for 10 sec and OFF for 20 sec and 0.15% final concentration of 5% PEI was added. The cell lysate was spun down by centrifugation at 21000 rpm at 4°C for 1 h. The supernatant was recovered for purification.

Column 1: Ni-Affinity Chromatography 5ml of 50 % Ni-IDA slurry was applied onto a 1.5 x 10 cm column. The column was first washed with deionised distilled H_2O, and then equilibrated with Binding buffer

Column 1 Buffers:
Lysis buffer: 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole 0.5mM TCEP
Wash buffer: 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 30mM Imidazole, 0.5mM TCEP
Elution buffer: 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 50 to 250mM Imidazole, 0.5mM TCEP

Column 1 Procedure: The supernatant was applied by gravity flow onto the Ni column. The Ni column was washed with Wash buffer and the bound protein was eluted by applying a step gradient of Imidazole (5 ml fractions of Elution buffer supplemented with 50mM, 100mM, 150mM and 250mM Imidazole). Collected fractions were pooled and stored at 4°C.

Growth Medium & Induction Protocol: A glycerol stock was used to inoculate a 50ml starter culture containing LB media with 50µg/ml Kanamycin and 34µg/ml Chloramphenicol. The starter culture was grown overnight at 37°C with shaking at 200 rpm. The following morning, three flasks containing 1L TB/Kanamycin were each inoculated with 10 ml of the starter culture. Cultures were incubated at 37°C with shaking at 180 rpm until an OD600nm = 0.8 was reached. The flasks were then cooled down to 18°C and 1mM IPGT was added to induce protein expression overnight. Cells were harvested by centrifugation at 5000 rpm at 4°C for 15 min. Cell pellets from each flask were resuspended in 15ml Binding buffer (50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole).

Column 2: Size Exclusion Chromatography - HiLoad 16/60 Superdex S75 (GE Healthcare)

Column 2 Buffers:
Gel Filtration buffer: 300mM NaCl, 50mM HEPES pH 7.5, 0.5mM TCEP

Column 2 Procedure: The Superdex S75 column was first equilibrated with Gel Filtration buffer. Concentrated the protein fraction from above step to <5ml using Vivaspin filter with a 10kDa cut-off, syringe injected onto the column and eluted with Gel Filtration buffer. Clean fractions containing the protein were pooled together.

Mass spec characterization: Pending analysis

Crystallization: Protein was buffered in 50mM HEPES pH 6, 300mM NaCl, 0.5 mM TCEP. Protein was concentrated to 15 mg/ml (calculated using extinction co-efficient of 26930). Native crystals were grown at 20°C in 150nl sitting drops mixing 50nl protein solution with 100nl of a reservoir solution containing 25% PEG 3350, 0.1M Bis-Tris pH 5.5. On mounting crystals were cryo-protected with an additional 25% Ethylene glycol.

Data Collection:
Resolution: 1.68Å **X-ray source**: Diamond IO3