Target ID: GLS2A
Entry Clone ID: GL2A-s002
Allele ID: GLS2A-a007
Construct ID GLS2A-c007
Clone ID GLS2A-k007
Expression ID GLS2A-e039
Purification ID: GLS2A-p005
Entry clone source: from collaborator
Vector: pNIC28-Bsa4
E.coli strain: BL21(DE3)-R3-pRARE2
Tags and additions: N-terminal, TEV protease cleavable hexahistidine tag

Coding DNA sequence:

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ATGCGCTCCATGAAGGCTCTGCAGAA
GGCCCTGAGCCGGGCTGGCAGTCACT
GCGGGCGAGGAGGCTGGGGTCACCCG
AGCCGGAGCCCCCTCCTTGGCGGGGG
CGTCCGGCACCACCTCAGTGAGGCCG
CGGCGCAGGGCAGAGAGACGCCACAC
AGCCACCAGCCGCAGCACCAGGATCA
TGATTCATCAGAAAGTGGCATGCTGT
CCCGCCTGGGTGATTTGCTCTTTTAC
ACTATTGCTGAAGGACAGGAACGAAT
CCCTATCCACAAGTTCACCACTGCAC
TAAAGGCCACTGGACTGCAGACATCA
GATCCTCGGCTCCGAGACTGCATGAG
CGAGATGCACCGCGTGGTCCAAGAGT
CCAGTAGTGGTGGCCTCTTGGACCGA
GATCTCTTCCGAAAGTGTGTGAGCAG
CAACATTGTGCTCCTGACCCAGGCAT
TCCGAAAGAAGTTTGTCATTCCTGAT
TTTGAGGAGTTCACGGGCCATGTGGA
TCGCATCTTTGAGGATGTCAAAGAGC
TCACTGGAGGCAAAGTGGCAGCCTAC
ATCCCTCAGCTGGCCAAGTCAAACCC
AGACCTGTGGGGTGTCTCCCTGTGCA
CTGTGGATGGTCAACGGCACTCTGTG
GGCCACACAAAGATCCCCTTCTGCCT
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ACTGACTACGTGCACAAGTTTGTGGG
CAAAGAGCCAAGTGGCCTGCGCTACA
ACAAGCTCTCCCTCAATGAGGAAGGA
ATCCCCCATAACCCCATGGTCAATGC
TGGTGCCATTGTTGTCAGCTCCCTGA
TCAAGATGGACTGTAACAAAGCAGAG
AAGTTTGATTTTGTGTTGCAGTATCT
CAACAAAATGGCTGGGAATGAATACA
TGGGTTTCAGCAATGCCACATTCCAG
TCAGAGAAGGAAACAGGGGATCGGAA
TTATGCCATCGGCTATTATCTCAAGG
AAAAGAAGTGCTTTCCTAAGGGGGTG
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GACATGATGGCTGCCCTTGATCTCTA
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CTTGTGAATCAGGCAGTGTCATGGCA
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CCCCATCACAGGCGAGAGTGTGCTGA
GTGCTGAAGCAGTGCGCAACACCCTC
AGCCTCATGCATTCCTGCGGCATGTA
TGACTTCTCTGGCCAGTTTGCCTTCC
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GTATCAGGAGCCATCCTCCTGGTGGT
ACCCAATGTCATGGGAATGATGTGCC
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CTGTGGTCAACCTGTTATTTGCTGCC
TATAGTGGCGATGTCTCAGCTCTTCG
AAGGTTTGCCTTGTCAGCCATGGATA
TGGAACAGAAAGACTATGACTCGCGC
ACAGCTCTGCATGTTGCTGCAGCTGA
AGGACACATCGAAGTTGTTAAATTCC
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TTTGCCAAGGACAGGTGGGGCAACAT
TCCCCTGGATGATGCTGTGCAGTTCA
ACCATCTGGAGGTGGTCAAACTGCTT
CAAGATTACCAGGACTCCTACACACT
CTCTGAAACTCAGGCTGAGGCAGCAG
CTGAGGCCCTGTCCAAAGAGAACTTA
GAAAGCATGGTATAG

Final protein sequence

mhhhhhhssgvdlgtenlyfq*sMIP
DFEEFTGHVDRIFEDVKELTGGKVAA
YIPQLAKSNPDLWGVSLCTVDGQRHS
VGHTKIPFCLQSCVKPLTYAISISTL
GTDYVHKFVGKEPSGLRYNKLSLNEE
GIPHNPMVNAGAIVVSSLIKMDCNKA
EKFDFVLQYLNKMAGNEYMGFSNATF
QSEKETGDRNYAIGYYLKEKKCFPKG
VDMMAALDLYFQLCSVEVTCESGSVM
AATLANGGICPITGESVLSAEAVRNT
LSLMHSCGMYDFSGQFAFHVGLPAKS
AVSGAILLVVPNVMGMMCLSPPLDKL
GNSHRGTSFCQKLVSLFNFHNYDNLR
HCARKLDPRREG

MHHHHHHSSGVDLGTENLYFQ*SM is the His6 tag (lower case) folowed by TEV protease recognition site (*).

Expression

Expression strain : BL21(DE3)-R3-pRARE2

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 TB media containing 50 Âµg/ml kanamycin and 50 Âµg/ml chloramphenicol, which was placed in a 37Â°C shaker overnight. The next day glycerol stocks were prepared from this overnight culture.

Expression

A glycerol stock was used to inoculate 100 ml of TB media containing 50 Âµg/ml kanamycin and 50 Âµg/ml chloramphenicol, which was placed in a 37Â°C shaker overnight. The next day this starter culture was used to inoculate 10L of TB media (7.5 ml starter culture used per 1L) containing 50 Âµg/ml kanamycin. When the OD600 reached approximately 0.8 the temperature was reduced to 18Â°C and after a further 1 hour the cells were induced by the addition of 0.1 mM IPTG. The expression was continued overnight.

Cell harvest

Cells were harvested by centrifugation at 6000 x g after which the supernatant was poured out and the cell pellet either placed in a -20Â°C freezer or used directly for purification.

Purification

Cell Lysis

Cell pellets were dissolved in approximately 50ml lysis buffer and broken by passing through a high pressure homogenizer at 15,000 psi for 4 cycles. The cell debris was pelleted at 35,000 x g and the supernatant used for further purification.

Lysis Buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole pH 7.5, 0.5 mM TCEP, 1 tablet per 50 ml protease inhibitor cocktail EDTA-free (Roche)

Column 1 Ni-NTA affinity

The clarified cell extract was collected and added to 1.5 mL of Ni-NTA in a 250 mL plastic bottle and kept in cold room under rotation for 1h30min. The resin was then washed in a glass column with Binding Buffer (40 ml) and Wash Buffer (30 ml). The protein was eluted with 25 ml of Elution Buffer in 5 x 5 ml fractions. All of these steps were done in cold room.

Binding Buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole pH 7.5, 0.5 mM TCEP

Wash Buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 40 mM Imidazole pH 7.5, 0.5 mM TCEP

Elution Buffer: 50 mM Hepes pH 7.5, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole pH 7.5, 0.5 mM TCEP

Column 2 Superdex 200 16/60 Gel Filtration

The first elution buffer fraction from column 1 was loaded in a Superdex 200 16/60 column (pre-equilibrated in GF Buffer) at 1.0 ml/min. 1.75 ml fractions were collected.

The protein was eluted at between 85 ml and 105 ml volume.

GF Buffer: 10 mM Hepes pH 7.5, 500 mM NaCl, 0.5 mM TCEP, 5% Glycerol.

Column 3 HiTrap Q HP Ion Exchange

Pooled fractions from gel filtration (elution volume around 85-105 ml) were concentrated to 5 mL, and diluted with Zero Salt Buffer to a final NaCl concentration of 50 mM. The diluted sample was loaded onto the Hitrap Q HP anionic exchange column that was pre equilibrated with Low Salt Buffer. Elution was performed with a linear NaCl gradient from 0% to 25% of High Salt Buffer in 187.5 ml. 1.75 mL fractions were collected. Protein was typically eluted at around 200 mM NaCl.

Zero Salt Buffer: 25 mM HEPES pH 8.0, 0.5 mM TCEP, 5% Glycerol.

Low Salt Buffer: 25 mM HEPES pH 8.0, 50 mM NaCl, 0.5 mM TCEP, 5% Glycerol.

High Salt Buffer: 25 mM HEPES pH 8.0, 2 M NaCl, 0.5 mM TCEP, 5% Glycerol.

Concentration

The purified protein was concentrated to 13 mg/ml using Millipore 10k mwco concentrators.

Mass spectrometry characterization

Measured mass: 38425.14 Da

Expected mass: 38426.2 Da

Crystallization

Crystals were grown by the sitting drop vapour diffusion method at 20Â°C. A sitting drop consisting of 100 nl protein and 50 nl well solution was equilibrated against well solution containing 0.1M tris pH 8.5, 0.2 M sodium chloride and 25%(w/v) PEG 3350. Crystals were mounted in the presence of 25 % (v/v) ethylene glycol and flash-cooled in liquid nitrogen.

Data collection

Resolution: 2.2 Ã□

X-ray source: Diamond Light Source beamline IO3