

GPT2

PDB:3IHJ

Revision

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Entry Clone Accession:BC062555

Entry Clone Source:Mammalian Gene Collection

SGC Clone Accession:GPT2A-k004

Tag:N-terminal hexahistidine tag with integrated TEV protease cleavage site:
mhhhhhhsgvd1gtenlyfq*sm

Host:BL21(DE3) R3 pRARE, where R3 denotes a derivative of BL21(DE3) resistant to a strain of T1 bacteriophage (SGC Oxford) and the pRARE plasmid originating from the Rosetta strain (Novagen) supplies tRNAs for rare codons.

Construct

Prelude:

Sequence:

mhhhhhhsgvd1gtenlyfq*smTLESMPQVKAVEYAVRGPIVLKAGEIELELQRGIKKPFTEVIRANIGDAQAMGQQPITFLRQ
VMACTYPNLLDSPSFPEDAKKRARRILQACGGNSLGSYSASQGVNCIREDVAAYITRRDGGVPADPDNIYLTTGASDGISTILKIL
VSGGGKSRTGVMIPIPQYPLYSAVISELDQVNYLDEENCWALNVNELRRAVQEAKDHCDPKVLCIINPGNPTGQVQSRKCIEDV
IHFAWEEKLFLLADEVYQDNVYSPDCRFHSFKVLYEMGPEYSSNVELASFHSTSKGYMGECGYRGGYMEVINLHPEIKGQLVKLLS
VRLCPPVSGQAAMDIVVNPPVAGEESFEQFSREKESVLGNLAKKAKLTEDLFNQVPGIHCNPLQGAMYAFPRIFIPAKAVEAAQAHQ
MAPDMFYCMKLLEETGICVVPGSGFGQREGTYHFRMTILPPVEKLKTVLQKVKDFHINFLEKYA

Vector:pNIC-Bsa4

Growth

Medium:Cells from a glycerol stock were grown in 20 ml TB supplemented with 8 g/l glycerol, 100 µg/ml kanamycin and 34 µg/ml chloramphenicol at 30 °C overnight. The overnight culture (20 ml) was used to inoculate 1.5 l TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin and approximately 200 µl 204 Antifoam A6426 (Sigma). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C until OD600 reached ~2. The culture was down-tempered to 18 °C over a period of 1 hour before target expression was induced by addition of 0.5 mM IPTG. Expression was allowed to continue overnight and cells were harvested the following morning by centrifugation (4,400 x g, 10 min, 4 °C). The resulting cell pellet (41 g wet cell weight) was resuspended in lysis buffer (1.5 ml/g cell pellet), supplemented with 2000 U Benzonase (Merck) and one tablet of Complete EDTA-free protease inhibitor (Roche Applied Science). The cell suspension was stored at -80 °C.

Antibiotics:

Procedure:

Purification

Procedure

Columns

IMAC: Ni-charged 2 ml HiTrap Chelating HP (GE Healthcare)

Gel filtration column: HiLoad 16/60 Superdex 200 Prep Grade (GE Healthcare)

Procedure

IMAC columns were equilibrated with IMAC wash1 buffer, and gel filtration columns were equilibrated with GF buffer. Purification of the protein was performed on an ÄKTAxpress system (GE Healthcare). The filtered lysate was loaded onto the Ni-charged HiTrap Chelating column and washed with IMAC wash1 buffer followed by IMAC wash2 buffer. Bound protein was eluted from the IMAC column with IMAC elution buffer and automatically loaded onto the gel filtration column. Fractions containing the target protein were identified by SDS-PAGE, pooled, and fresh TCEP was added to a final concentration of 2 mM. The protein was concentrated using an Amicon Ultra-15 centrifugal filter device (10,000 NMWL; Millipore) to 17 mg/ml in a volume of 0.8 ml. The identity of the protein was confirmed by mass spectrometry.

Extraction

Procedure

The cell suspension was quickly thawed in water. Cells were disrupted by sonication (Vibra-Cell, Sonics) at 80% amplitude for 3 min effective time (pulsed 4s on, 4s off) and cell debris was removed by centrifugation (49,000 x g, 20 min, 4 °C). The supernatant was decanted and filtered through a 0.45 µm flask filter.

Concentration:

Ligand

MassSpec:

Crystallization: Crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. 0.2 µl protein solution (17 mg/ml) was mixed with 0.1 µl of well solution consisting of 0.1 M citric acid pH 4.2, 0.1 M disodium hydrogen phosphate, 40% PEG 300. The plate was incubated at 4 °C. For data collection the crystals were quickly transferred to a cryo solution consisting of well solution complemented with 20% glycerol, and flash frozen in liquid nitrogen.

NMR Spectroscopy:

Data Collection: Diffraction data to 2.3 Å resolution was collected at DIAMOND (I03)

Data Processing: The structure was solved by molecular replacement using alanine aminotransferase from Pyrococcus furiosus as a template (PDB: 1XI9). The space group was P6122 with cell dimensions $a=92.73$ Å, $b=92.73$ Å, $c=262.87$ Å, $\alpha=90^\circ$, $\beta=90^\circ$, $\gamma=120^\circ$. Two monomers were located in the asymmetric unit. The structure was refined with REFMAC5. Data in the interval 19.73-2.3 Å resolution was used and at the end of the refinement the R values were: $R=19.2\%$ and $R_{free}=23.8\%$. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 3IHJ.