

DDX20 + AMPPNP

PDB:3B7G

Revision

Revision Type:created

Revised by:created

Revision Date:created

Entry Clone Accession:gi|23270929

Entry Clone Source:Mammalian Gene Collection

SGC Clone Accession:DDX20A-s001

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

mhahhhhhsgvd1gtenlyfq*sm

Host:*E.coli* BL21-Gold(DE3)pRARE2, where BL21-Gold(DE3) cells (Stratagene) have been transformed with pRARE2 originating from the Rosetta2 strain (Novagen). The pRARE2 plasmid supplies tRNAs for rare codons.

Construct

Prelude:

Sequence:

mhahhhhhsgvd1gtenlyfq*smRTAQDLSSPRTGVDVLLAEPADFESLLLSPVLEGLRAAGFERPSPVQLKAIPLGRCGLDLI
VQAKSGTGKTCVFSTIALDSLVLENLSTQILILAPTRTREIAVQIHSVITAIGIKMEGLECHVFIGGTPSQDKTRLKKCHIAVGSPGR
IKQLIELDYLNPGSIRLFILDEADKLLEEGSFQEIQINWIYSSLPASKQMLAVSATYPEFLANALTKYMRDPTFVRLNS

Vector:pNIC-Bsa4

Growth

Medium:

Antibiotics:

Procedure: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 BREOX FMT 30 anti-foam solution (Cognis Performance Chemicals UK Ltd). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C until OD600 reached ~1.7. 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 (5,500 x g, 10 min, 4 °C). The resulting cell pellet (31.8 g wet cell weight) was resuspended in lysis buffer (1.5 ml/g cell pellet), supplemented with one tablet of Complete EDTA-free protease inhibitor (Roche Applied Science). The cell suspension was stored at -80 °C.

Purification

Procedure

Columns

IMAC: Ni-charged 1 ml HiTrap Chelating HP (GE Healthcare)

Gel filtration column: HiLoad 16/60 Superdex 75 Prep Grade (GE Healthcare)

Procedure

Purification of the protein was performed as a two step process on an ÄKTAxpress system (GE Healthcare). Prior to purification, columns were equilibrated with IMAC wash1 buffer and gel filtration buffer, respectively. 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 pooled and fresh TCEP was added to a final concentration of 2 mM.

Tag removal

The N-terminal histidine tag was proteolytically removed by incubating the target protein with His-tagged TEV protease at a molar ratio of 50:1 at 4 °C overnight. The proteolytic reaction did not reach completion, as judged by SDS-PAGE. Target protein was purified from uncleaved protein, tag and protease by passing the reaction mixture over a Ni-charged 1 ml HisTrap FF column (GE Healthcare) pre-equilibrated with GF buffer (500 mM NaCl and 10 mM imidazole). The cleaved protein was concentrated and the buffer was exchanged to 20 mM HEPES, 500 mM NaCl, 10% glycerol, 2 mM TCEP, pH 7.5, using a Vivaspin 20 centrifugal filter device with 10,000 MWCO (Sartorius). The final protein concentration was determined to 33.8 mg/ml in a volume of 0.17 ml and the identity of the protein was confirmed by mass spectrometry.

Extraction

Procedure

The cell suspension was quickly thawed in water and 2000 U Benzonase (Merck) were added. 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 (49,100 x g, 20 min, 4 °C). The supernatant was decanted and filtered through a 0.45 µm flask filter.

Concentration:

Ligand

AMPPNP Mass Spec:

Crystallization: The crystal were obtained by the sitting drop vapour diffusion method in a 96-well plate. 0.1 µl protein solution (diluted to 20 mg/ml) including 20 mM ADP and 10 mM MgCl₂ was mixed with 0.1 µl of well solution consisting of 0.1 M bis-Tris, pH 5.5, 0.2 M NaCl and 12% PEG 3350. The plate was incubated at 4 °C and crystals appeared after one day and continued to grow for one more week. The crystal was briefly transferred to a cryo solution consisting of well solution complemented with 28% glycerol, and flash frozen in liquid nitrogen.

NMR Spectroscopy:

Data Collection:

Data Processing: