RBBP4

PDB:3GFC

Revision

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Entry Clone Accession:NP_005601
Entry Clone Source:MGC AU12-D2
SGC Clone Accession:
Tag:N-terminal tag: MGSSHHHHHHSSGLVPRGS
Host:SF9 insect cells

Construct

Prelude:
Sequence:

mgsshhhhhhssglvprgsMADKEAAFDDAVEERVINEEYKIWKKNTPFLYDLVMTHALEWPSLTAQWLPDVTRPEGKDFSIHRLVL
GTHTSDEQNHLVIASVQLPNDDAQFDASHYDSEKGEFGGFGSVSGKIEIEIKINHEGEVNRARYMPQNPCIIATKTPSSDVLVFDYT
KHPSKPDPSGECNPDLRLRGHQKEGYGLSWNPNLSGHLLSASDDHTICLWDISAVPKEGKVVDAKTIFTGHTAVVEDVSWHLLHESL
FGSVADDQKLMIWDTRSNNTSKPSHSVDAHTAEVNCLSFNPYSEFILATGSADKTVALWDLRNLKLKLHSFESHKDEIFQVQWSPHN
ETILASSGTDRRLNVWDLSKIGEEQSPEDAEDGPPELLFIHGGHTAKISDFSWNPNEPWVICSVSEDNIMQVWQMAENIYNDEDPEG
SVDPEGQGS

Vector:pFBoc-lic

Growth

Medium:
Antibiotics:
Procedure:Transposition: 2 µL of the construct was added and mixed to 30 µl of DH10Bac competent cells in a sterile 96-well microtitre plate on ice. The plate was left on ice for a further 30 minutes. The heat-shock procedure was done by transferring the plate to a 42 °C water bath for 60 seconds and then returning it to ice for a further 2 minutes. 600 µl of SOC medium (pre-warmed to 37°C) was added to the well and the plate incubated at 37°C for 5 hours. The 2 µl culture mixed with pre-warmed 100 µl SOC, and plated out onto LB agar in a 5.5 cm Petri dish contains Gentamicin (7 µg/mL), Kanamycin (50 µg/mL) and Tetracycline (10 µg/mL), Bluo-gal (200 µg/ml), and IPTG (40 µg/ml). The plates were incubated at 37°C for 48 hours.

Bacmid preparation: One white colony was picked into 3 mL of LB media, with Gentamicin (7 µg/mL), Kanamycin (50 µg/mL) and Tetracycline (10 µg/mL), in a 24-well block (Qiagen, Cat. 19583) and placed in a shaker (250 rpm) for 18 hours at 37°C. Bacmids were purified with Montage(R) kit (Millipore Cat. LSKB09604).

Generation of P1 recombinant Baculovirus: In a Napflow(R) Class II type A/B3 biosafty

cabinet, 50 µl HyQ(R) SFX-insect serum medium (Hyclone, Cat. SH30278.02) was added into 6 µg bacmid and 3ul cellfectin (Invitrogen Cat. 10362-010). Then bacmid and cellfactin in the medium were mixed and incubated at room temperature for 45 minutes. 1 mL SF9 cells (2 x 105 cells/mL) in HyQ® SFX-insect serum medium was added into the mixture in a 24 well plate (Falcon Cat. 353047). After cells sat at the bottom of the plate, remove supernatant, and 280 µl HyQ® SFX-insect serum medium was added to the plate, then the plate was incubated at 27 °C for 5 hours. In the plate, the supernatant of the mixture was replaced with 0.7 mL Graces insect medium (Invitrogen Cat. 11595-030) contained 10% FBS (Invitrogen Cat.12483-020) and 1% antibiotics (100 µg/mL penicillin, 100 µg/mL streptomycin). Then the plate was incubated in 27 °C for 72 hours. The supernatant was collected.

Generation of P2 recombinant Baculovirus: In a 6 well plate (Falcon Cat. 353047), SF9 cells (1 x 106 cells / mL) in 1.5 mL HyQ® SFX-insect serum medium were infected with 80 µl P1 viruses in 27 °C. The culture was incubated in 27 °C for 48 ~ 72 hours. Supernatant was collected after incubation.

Generation of P3 recombinant Baculovirus: In a 500 mL flask, sf9 cells were added into HyQ® SFX-insect serum medium to reach the density of 2 x 106 cells / mL. 0.2 mL of P2 recombinant Baculovirus was added into the 200ml culture. The flask was shaken in 27 °C, 130 rpm for 48 hours. Supernatant was collected.

Protein production: 5-10 mL P3 recombinant Baculovirus cells were added into 1 L HyQ® SFX-insect serum medium contained High-Five cells (2 x 106 cells / mL) and Gentamicin (10 µg / mL). The culture was put on a shaker with 100 rpm, at 27 °C for 48 hours. Cells were harvested with centrifuge (4000 rpm, 15 minutes). Harvested cells were washed with cold PBS buffer, then flash frozen in liquid nitrogen and stored at -80 °C.

Purification

Procedure
IMAC: Unclarified lysate was mixed with 2-3 mL of Ni-NTA superflow Resin (Qiagen) per 40 mL lysate. The mixture was incubated with mixing for at least 45 minutes at 4oC. The mixture was than loaded onto an empty comlum (BioRad) and washed with 100 ml wash buffer. Samples were eluted from the resin by exposure to 2-3 column volumes (approx. 10-15 mL) of elution buffer. Concentration of eluted protein was estimated by OD280

Gel filtration chromatography: An XK 26x65 column (GE Healthcare) packed with HighLoad Superdex 75 resin (GE Healthcare) was pre-equilibrated with gel filtration buffer for 1.5 column volumes using an AKTA explorer (GE Healthcare) at a flow rate of 2.5mL/min. The Eluted sample from the IMAC step (approx. 15 mL) was loaded onto the column at 1.5 mL/min, and 2mL fractions were collected into 96-well plates (VWR 40002-012) using peak fractionation protocols). Fractions observed by a UV absorption chromatogram to contain the protein were pooled.

Extraction

Procedure
Frozen cell pellet were thawed and protease inhibitors cocktail (Roche) and 0.1% NP40 (final concentration) were added. Cell lysis was accomplished by sonication (Virtis408912, Virsonic) on ice: the sonication protocol was 10 sec pulse at half-maximal frequency (5.0), 10 second rest,

for 10 minutes total sonication time per pellet.
Concentration: Purified proteins were concentrated using 15 mL concentrators with a 5,000 molecular weight cut-off (Amicon Ultra-15, UFC900524, Millipore) at 3750 rpm, typically resulting in a final concentration around 20 mg/mL.
Ligand
MassSpec:
Crystallization: Diffraction quality crystals were grown using the following protocol: 25% PEG3350, 0.2M MgCl2, 0.1M HEPES, pH7.5, vapor diffusion, sitting drop, temperature 297k
NMR Spectroscopy:
Data Collection:
Data Processing: