KIF9

PDB:2NR8

Revision

Revision Type:created
Revised by:created
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Entry Clone Accession:gi:34193691
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SGC Clone Accession:
Tag:
Host: Hi-Five insect cells.

Construct

Prelude:
Sequence:

mgsshhhhhhssglvprgsGTRKKVHAFVRVKPTDDFAHEMIRYGDDKRSIDIHLKKDIRRGVVNNQQTDWSFKLDGVLHDASQDLV
YETVAKDVVSQALDGYNGTIMCYGQTGAGKTYTMMGATENYKHRGILPRALQQVFRMIEERPTHAITVRVSYLEIYNESLFDLLSTL
PYVGPSVTPMTIVENPQGVFIKGLSVHLTSQEEDAFSLLFEGETNRIIASHTMNKNSSRSHCIFTIYLEAHSRTLSEEKYITSKINL
VDLAGSERLGKSGSEGQVLKEATYINKSLSFLEQAIIALGDQKRDHIPFRQCKLTHALKDSLGGNCNMVLVTNIYGEAAQLEETLSS
LRFASRMKLV

Vector:pFBOH-LIC

Growth

Medium:
Antibiotics:
Procedure: Transposition: 2 microL 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 degC water bath for 60 seconds and then returning it to ice for a further 2 minutes. 600 µl of SOC medium (pre-warmed to 37degC) 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). The plates were incubated at 37degC 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 37degC. 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 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 Grace?s 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, high-five cells were added into HyQ® SFX-insect serum medium to reach the desity of 2 x 106 cells / ml. 0.2 ml of P2 recombinant Baculovirus was added into the culture. The flask was shaken in 27 °C, 130 rpm for 48 hours. Supernatant was collected.
Protein production: 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, 20 minutes). Harvested cells were washed with cold PBS buffer, then flash frozen in liquid nitrogen and stored at -80 degC.

Purification

Procedure
Column 1: Ni-NTA beads

Column 2: Size exclusion chromatography (Superdex 200 26/60)
The fractions eluted of the Ni-affinity chromatography applied to a Superdex S200 column equilibrated in SEC buffer at a flow rate of 2.0 ml/min. Eluted fractions were 95% pure as judged by SDS-PAGE.Concentration:Centricon with a 10kDa cut off in SEC-buffer. Protein concentrations were measured using Bradford assay with purity >95% based on SDS-PAGE analysis.

Extraction

Procedure
50 mM Hepes, pH 8.0, 0.5 M NaCl, 5% glycerol (v/v), and 5 mM imidazole, 1 mM TCEP. Six EDTA-free Complete® protease inhibitor tablets in 100 ml of extraction buffer, 1.2 ml of 0.05 M PMSF, 1.2 ml 0.1 M benzamidine and 3750 units of benzonase were added in to extraction buffer. 10g pellets / 100 ml extraction buffer. Cell breakage: 1 passes through the Emulsiflex C5 high pressure homogeniser. Centrifuge for 60 mins at 16000 rpm and 4 °C to remove cell debris. Discard pellet.
Concentration:
Ligand
MassSpec:
Crystallization: Crystals were obtained using the vapor diffusion method and a protein concentration of 16 mg/ml containing 5 molar fold of ADP and MgCl2. 0.5 µl of the concentrated protein mixed with 0.5 µl of a well solution containing 1.5 M (NH4)2SO4, 0.1 M Tris, pH 8.50. Crystals appeared after a day at 18 °C.
NMR Spectroscopy:
Data Collection: Resolution: 2.0Å, X-ray source: Crystals were cryo-protected using 50% mineral oil and 50% parotone, and flash frozen in liquid nitrogen. Diffraction data were collected at the home source to 2.0Å.
Data Processing: