PLXNB1

PDB:3HM6

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:pGEX-6P-1-Myc-PLXNB1-Y1511-L2135, cDNA equivalent to GenBank:X87904.2
Entry Clone Source: Dr. Manabu Negishi (Kyoto Univ., Japan)
SGC Clone Accession:clone pkey: 38412 HPC072-F10
Tag:mgsshhhhhhssglvpr*gs
Host:SF9 cell, Spodoptera frugiperda

Construct

Prelude: Tag not removed
PLXNB1:Y1511-L2135
Sequence:

mgsshhhhhhssglvprgsYRRKSKQALRDYKKVQIQLENLESSVRDRCKKEFTDLMTEMTDLTSDLLGSGIPFLDYKVYAERIFFP
GHRESPLHRDLGVPESRRPTVEQGLGQLSNLLNSKLFLTKFIHTLETQRTFSARDRAYVASLLTVALHGKLEYFTDILRTLLSDLVA
QYVAKNPKLMLRRTETVVEKLLTNWMSICLYTFVRDSVGEPLYMLFRGIKHQVDKGPVDSVTGKAKYTLNDNRLLREDVEYRPLTLN
ALLAVGPGAGEAQGVPVKVLDCDTISQAKEKMLDQLYKGVPLTQRPDPRTLDVEWRSGVAGHLILSDEDVTSEVQGLWRRLNTLQHY
KVPDGATVALVPCLTKHVLRENQDYVPGERTPMLEDVDEGGIRPWHLVKPSDEPEPPRPRRGSLRGGERERAKAIPEIYLTRLLSMK
GTLQKFVDDLFQVILSTSRPVPLAVKYFFDLLDEQAQQHGISDQDTIHIWKTNSLPLRFWINIIKNPQFVFDVQTSDNMDAVLLVIA
QTFMDACTLADHKLGRDSPINKLLYARDIPRYKRMVERYYADIRQTVPASDQEMNSVLAELSWNYSGDLGARVALHELYKYINKYYD
QIITALEEDGTAQKMQLGYRLQQIAAAVENKVTDL

Vector:pFBOH-LIC (GI:134105591)

Growth

Medium: HyQ SFX-insect
Antibiotics:
Procedure:Transposition: 2 µL plasmid DNA was added and mixed to 30 µL DH10Bac competent cells in a sterile 96-well microtitre plate on ice. The plate was left on ice for a further 30 minutes. The cells were heat-shocked in a 42 degC water bath for 60 seconds and then chilled on ice for 2 minutes. 600 µL SOC medium (pre-warmed to 37 degC) was added to the cells and the plate was incubated at 37 degC for 5 hours. 2 µL 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), blue-gal. The plates were incubated at 37 degC 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 degC. Bacmids were purified with

Montage9(R) kit (Millipore Cat. LSKB09604).

Generation of P1 recombinant Baculovirus: In a Napflow(R) Class II type A/B3 biosafty cabinet, 50 µL HyQ SFX-insect serum-free 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 ($2x10^5$ 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 degC 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 degC for 72 hours. The supernatant was collected.

Generation of P2 recombinant Baculovirus: In a 6 well plate (Falcon Cat. 353047), SF9 cells ($1 x 10^6$ cells/mL) in 1.5 mL HyQ SFX-insect serum medium were infected with 80 µL P1 viruses in 27 degC. The culture was incubated in 27 degC 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 density of $2x10^6$ cells / mL. 0.2 mL of P2 recombinant Baculovirus was added into the culture. The flask was shaken in 27 degC, 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 ($2x10^6$ cells / mL) and Gentamicin (10 µg/mL). The culture was put on a shaker with 100 rpm, at 27 degC 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
The Parr Bomb treated lysate were centrifuged at 15,500 rpm (36,000xg) for one hour and the supernatant were aliquoted into 3x 50 mL Falcone tubes. Each was added with 0.5 mL fresh 50% Ni-NTA beads (pre-exchanged in 50mM Tris pH 7.5 buffer) and kept on a rotary drum fro 1.5 hrs at 4 degC.

The aliquots were then centrifigued at 1250xg for 3 minutes. The beads were pooled into one tube, and washed with 15 mL buffer B once, 15 mL buffer W once. Bound proteins were eluted from the beads with 8mL followed by 7mL Buffer E. The elutant was combined and loaded onto an SD200 26/60 gel filtration column. Fractions containing target protein were pooled and concentrated using Amicon centrifugal concentrator (m.w. cut-off 30,000) to a final concentration of 6.9 mg/mL. The purity of the concentrated protein was higher than 95% judging from SDS-PAGE.

Extraction

Procedure
Frozen cells from 2.4L culture were thawed on ice and supplemented with 1mL Protease Inhibitor

cocktail (Sigma-Aldrich), 8uL Benzonase nuclease, 1 mL PMSF/Benzamide stock solution, 10 mL 10% NP-40 detergent. Buffer B was added to a final volume of 150mL, cells were fully resuspended by blender for 5 seconds, and cells were lysed by treating with Parr Bomb for 15 minutes on ice at 1500 psi.
Concentration: Native 6.94 mg/mL, Bradford measurement
SeMet 3.46 mg/mL, Bradford measurement
Ligand
MassSpec: Native: 73459.06, expected 73541.32 or 73410.12 (without the N-term Met)
SeMet: 74020.86.
Crystallization: Crystal used for data collection were grown in 16% PEG2000 MME, 0.1 M Tris pH 8.0, 0.2 M TMAO(Trimethylamine N-oxide) in hanging drop setup. 1uL protein solution were mixed with 1uL well solution. The crystals usually appear in two to three days. Phase information was obtained from a mercury-derivative of native plexin B1 crystal. To make the mercury-derivative, native plexin B1 crystals were soaked in the well solution supplemented with 1mM thiomersal for 6 hours and then flash freezed for data collection.
Cryoprotectant: well solution plus 20% EG for native, 20% PEG3350 + 20% EG for Hg derivative.
NMR Spectroscopy:
Data Collection:
Data Processing: