ZFYVE9C

PDB:4BKW

Revision

Revision Type:created
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Entry Clone Accession:GI: BC032680
Entry Clone Source:MGC
SGC Clone Accession:ZFYVE9C-c009
Tag:MGHHHHHHSSGVDLGTENLYFQ. N-terminal hexahistidine tag cleavable by TEV protease.
Host:BL21 (DE3)-R3-pRARE2. Phage-resistant derivative of BL21 (DE3), with pRARE2 plasmid encoding rare codon tRNAs (chloramphenicol-resistant).

Construct

Prelude:
Sequence:

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MHHHHHHSSGVDLGTENLYFQSMNLIPEDGLPPILISTGVKGDYAVEEKPSQISVMQQLEDGGPDPLVFVLNANLLSMVKIVNYVNR
KCWCFTTKGMHAVGQSEIVILLQCLPDEKCLPKDIFNHFVQLYRDALAGNVVSNLGHSFFSQSFLGSKEHGGFLYVTSTYQSLQDLV
LPTPPYLFGILIQKWETPWAKVFPIRLMLRLGAEYRLYPCPLFSVRFRKPLFGETGHTIMNLLADFRNYQYTLPVVQGLVVDMEVRK
TSIKIPSNRYNEMMKAMNKSNEHVLAGGACFNEKADSHLVCVQNDDGNYQTQAISIHNQPRKVTGASFFVFSGALKSSSGYLAKSSI
VEDGVMVQITAENMDSLRQALREMKDFTITCGKADAEEPQEHIHIQWVDDDKNVSKGVVSPIDGKSMETITNVKIFHGSEYKANGKV
IRWTEVFFLENDDQHNCLSDPADHSRLTEHVAKAFCLALCPHLKLLKEDGMTKLGLRVTLDSDQVGYQAGSNGQPLPSQYMNDLDSA
LVPVIHGGACQLSEGPVVMELIFYILENIV
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Vector:pNIC28-Bsa4. T7/lac regulated, N-terminal His-tag, TEV, LIC cloning using BsaI cleavage/T4 polymerase, SacB stuffer fragment, pET28 backbone.

Growth

Medium:A glycerol stock was used to inoculate a 100ml starter culture containing LB media with 50Âµg/ml Kanamycin and 34Âµg/ml Chloramphenicol. The starter culture was grown overnight at 37Â°C with shaking at 200 rpm. The following morning, two flasks containing 1L LB/Kanamycin were each inoculated with 10 ml of the starter culture. Cultures were incubated at 37Â°C with shaking at 170 rpm until an OD600nm ≥ 0.4 was reached. The flasks were then cooled down to 18Â°C to an OD600nm ≥ 0.7 and added 0.4mM IPTG to induce protein expression overnight. Cells were harvested by centrifugation at 5000 rpm at 4Â°C for 15 min. Cell pellets from each flask were resuspended in 25ml Binding buffer (50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole).
Antibiotics:
Procedure:

Purification

Buffers
Procedure

Extraction

Buffers
Procedure
Extraction buffer, extraction method: The frozen cells were thawed. The cells were lysed by ultrasonication over 15 min with the sonicator pulsing ON for 5 sec and OFF for 10. A final concentration of 0.15% PEI was added to the lysate. The cell lysate was spun down by centrifugation at 21K rpm at 4°C for 1 h. The supernatant was recovered for purification. Column 1: Ni-Affinity Chromatography. 4 ml of 50 % Ni-sepharose slurry was applied onto a 1.5 x 10 cm column. The column was equilibrated with binding buffer (25ml). Buffers: Binding buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole 0.5mM TCEP Wash buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 25 mM imidazole 0.5mM TCEPElution buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% Glycerol; 50 and 250 mM imidazole 0.5mM TCEPProcedure: The supernatant following centrifugation was applied by gravity flow onto the Ni-sepharose column. The bound protein was then washed with 50ml binding buffer and subsequently with 30 ml wash buffer. ZFYVE9C protein was then eluted by applying a step gradient of imidazole Â using 5 ml portions of elution buffer with increasing concentration of imidazole (1 x 50 mM, 3 x 250 mM). Elution fractions were analyzed by SDS PAGE and the 3 x 250 mM imidazole fractions were kept and pooled. 10 mM DTT was added for overnight storage at 4□C.Enzymatic treatment: TEV protease cleavage. Fractions containing ZFYVE9C were treated with TEV protease overnight at 4°C.Column 2: Size Exclusion Chromatography Â S200 HiLoad 16/60 Superdex run on ÄKTA-ExpressGel Filtration buffer: 300 mM NaCl, 50 mM HEPES pH 7.5, 0.5 mM TCEP, pH 7.5Procedure: The Superdex S200 column was first equilibrated with Gel Filtration buffer. The protein fraction from above step was concentrated to <5ml using Vivaspin filter with a 30kDa cut-off, before being syringe injected onto the column and eluted with Gel Filtration buffer. Clean fractions containing the protein were pooled together.Column 3: Cation Exchange Chromatography. HiTrap QP HP 1ml columnBuffer: QA Buffer I: 50mM HEPES, 50mM NaCl pH 8QB Buffer II: 1M NaCl, 50mM HEPES pH 8Procedure: The HiTrap QP HP column was first washed with QB buffer II and then equilibrated with QA buffer I. The protein fraction from above step was concentrated to 500µl using an Amicon Ultra-15 filter with a 30kDa cut-off. The 500µl fraction was made up to 10ml with QA buffer I and applied onto the column manually using a syringe. Bound protein was eluted in 0%-100% gradient with QB buffer II. Clean fractions containing the protein were pooled together.
Concentration:
Ligand
MassSpec:
Crystallization: Protein was concentrated down to 500ul and diluted to 10ml in gel filtration buffer before being reconcentrated down to 12.6mg/ml. Crystals were grown at 20°C in 300nl sitting drops mixing 200nl protein solution with 100nl reservoir solution containing 30% PEG 3350; 0.1M bis-tris pH6.5; 0.15M magnesium chloride. On mounting crystals were cryo-protected with an additional 25% ethylene glycol.
NMR Spectroscopy:
Data Collection:2.53 Ã□ resolution; X-ray source: Diamond Light Source, station I03, using monochromatic radiation at wavelength 0.9795 Ã□
Data Processing: