Entry Clone Source: FivePrime

Entry Clone Accession: NM_014720 Variant

SGC Construct ID: SLKA-c004

GenBank GI number: gi|41281453

Entry clone accession: gi|41281453

Vector: pNIC28-Bsa4. Details [PDF]; Sequence [FASTA] or [GenBank]

Tags and additions: Tag sequence:
mhhhhhhssgvdlgtenlyfq*s(m) TEV-cleavable (*) N-terminal his6 tag.

Final protein sequence:
mhhhhhhssgvdlgtenlyfqsmKQYEHV
TRDLNPEDFWEIIGELGDGAFGKVYKAQN
KETSVLAAAKVIDTKSEEELEDYMVEIDI
LASCDHPNIVKLLDAFYYENNLWILIEFC
AGGAVDAVMLELERPLTESQIQVVCKQTL
DALNYLHDNKIIHRDLKAGNILFTLDGDI
KLADFGVSAKNTRTIQRRDSFIGTPYWMA
PEVVMCETSKDRPYDYKADVWSLGITLIE
MAEIEPPHHELNPMRVLLKIAKSEPPTLA
QPSRWSSNFKDFLKKCLEKNVDARWTTSQ
LLQHPFVTVDSNKPIRELIAEAKAEVTEE
VEDGKE

The highlighted residue (red) corresponds to mutation (K/T) with respect to the reference sequence

Host: BL21 (DE3)

Growth medium, induction protocol: 1ml from a 10 ml overnight culture (BL21 Rosetta strain) containing 50 µg/ml kanamycine and 35 µg/ml chloramphenicole was used to inoculate 1 liter of LB media containing the same concentration of antibiotics. Cultures were grown at 37°C until the OD_{600} reached ~0.3. After that the temperature was adjusted to 20°C. Expression was induced for 4 hours using 1mM IPTG at an OD_{600} of 0.8. The cells were collected by centrifugation and the pellet resuspended in binding buffer and frozen. **Binding buffer:** 50mM HEPES pH 7.5; 300 mM NaCl; 20 mM imidazole.

Extraction buffer, extraction method: Cell pellets were lysed by sonication. The lysate was centrifuged at 19,000 rpm for 60 minutes and the supernatant collected for purification.

Column 1: Ni-affinity chromatography.

Buffers: Binding buffer: 50 mM HEPES pH 7.5, 300mM NaCl,, 20 mM Imidazole. **Wash buffer 1:** 50 mM HEPES pH 7.5, 1M NaCl, 20mM Imidazole. **Wash buffer 2** : as for lysis buffer. **Elution buffer:** 50mM HEPES pH 7.5, 300mM NaCl, 150 mM Imidazole.

Procedure: 5 ml of 50% Ni-NTA slurry (Qiagen) was applied to a 1.5 x 10 cm gravity column. The column was equilibrated with 50 ml binding buffer. The lysate was applied to the column which was subsequently washed with 50 ml wash buffer 1 and 2. SLK was eluted with 25 mls of elution buffer. The eluted protein was collected and analyzed by SDS-PAGE. DTT was added to the protein sample to a final concentration of 10mM. The N-terminal his 6 -tag was cleaved by incubating the protein overnight with TEV protease.

Column 2: Size exclusion chromatography (Superdex S75, 60 x 1cm)

SEC-Buffers: 50 mM Hepes, pH 7.5, 300 mM NaCl, 5 mM DTT.

Procedure: The fractions eluted of the Ni-affinity chromatography were concentrated to about 4 mls using Centricon concentrators (10kDa cut off). The concentrated protein was applied to a Superdex S75 column equilibrated in SEC buffer at a flow rate of 0.8 ml/min. Eluted fractions were 95% pure as judged by SDS-PAGE.

Protein concentration: Centricon with a 10kDa cut off in SEC-buffer

Crystallization: Crystals were obtained using the vapor diffusion method and a protein concentration of 10 mg/ml containing 1 mM Cdk1/2 Inhibitor III (CalbioChem) by mixing 100nl of the concentrated protein with 50nl of a well solution containing 16% PEG3350, 0.15M KSCN, 0.1M BisTris propane pH 6.5 and 10 % ethylene glycol. Crystals of the (4-(4-(5- cyclopropyl- 1H-pyrazol-3- ylamino)-quinazolin-2-ylamino)-3 phenyl)-acetonitrile complex were obtained using essentially mono-phosphorylated protein a protein concentration of 10 mg/ml containing 1 mM of the inhibitor by mixing 100 nl of protein solution with 50 nl of a buffer containing 18% PEG3350, 0.15M KSCN, 0.1M BisTris propane pH 6.5 and 10 % ethylene glycol. Crystals appeared after a couple of days at 4°C.

Data Collection: Crystals were cryo-protected using the well solution supplemented with an additional 15% ethylene glycol and flash frozen in liquid nitrogen. Diffraction data were collected at the SLS beam line X10 (λ=0.979 Å) to 2.1 / 2.3Å