

Entry Clone Source: MGC
Entry Clone Accession: IMAGE:4284122
SGC Construct ID: RAC3A-c012
GenBank GI number: gi 4826962
Vector: pNIC28-Bsa4. Details [PDF]; Sequence [FASTA] or [GenBank]
Tags and additions: N-terminal hexahistidine tag with a TEV cleavage site
<p>Sequence: This is a mutant form of RAC 3 with the Cys178 replace by Gly. The Cys178Gly mutation is indicated in red.</p> <p>mhhhhhssgvdlgtenlyfq*smQAIKC VVVGDAVGKTCLLISYTTNAFPGEYIPT VFDNYSANVMVDGKPVNLGLWDTAGQEDY DRLRPLSYPQTDVFLICFSLVSPASFENV RAKWYPEVRHHCPTHPIILLVGTKLDLRDD KDTIERLRDKKLAPITYPQGLAMAREIGS VKYLECSALTQRGLKTVFDEAIRAVLG</p> <p>Residues in lower case are the histidine tag followed by the TEV recognition sequence with the cleavage site marked with a *.</p>
Host : BL-21(DE3)R3
Growth medium, induction protocol: Freshly transformed <i>E. coli</i> cells was used to inoculate 1 litre of TB plus 100 µg/ml ampicillin. When OD ₆₀₀ reached ~0.5 the temperature was shifted down from 37°C to 25°C for 1 hour before induction with the addition of 1 mM IPTG. Protein expression was allowed to carry on for a further 4 hours before harvest.
Extraction buffer, extraction method: All extraction steps were carried out at 4°C.
Extraction buffer (EX): 50 mM Hepes pH 8.0, 150 mM NaCl, 5 % Glycerol, 10 mM Imidazole pH 8.0, 10 mM MgCl ₂ . 1 tablet proteinase inhibitor in 10ml EX buffer was added to the 1L growth pellet. Total vol: 45 mls (estimate). Cell breakage: 5 passes through the Emulsiflex C5 high pressure homogeniser. Total vol: 50 mls (estimate). Centrifuge for 40 mins at 16000 rpm and 4°C to remove cell debris. Discard pellet.
Column 1 : Low pressure chromatography using Bio-Rad Econo column (2.5 cm x 13 cm).
Buffers: Wash buffer I (WB1): 50 mM Tris pH 8.0, 150 mM NaCl, 5% Glycerol, 10mM MgCl ₂ , 10mM Imidazole pH 8.0; Wash Buffer II (WBII): 50 mM Tris pH 8.0, 150mM NaCl, 5% Glycerol, 10mM MgCl ₂ , 30mM Imidazole pH 8.0; Elution buffer (EB): 50 mM Tris pH 8.0, 150 mM NaCl, 5% Glycerol, 10mM MgCl ₂ , 250mM Imidazole pH 8.0.
<p>Procedure: Total volume of Ni-NTA added to BioRad drip column: 4 mls (50%).</p> <p>Resin washed with 12.5 ml of WB1. The supernatant was applied to a column using 5 ml pipette and allowed to pass over the resin. The flow through was collected in a 50 ml falcon tube and applied once more to the column. Two wash steps followed. Wash with 12.5 ml of WBI. Wash with 12.5 ml column vols of WBII. Elute with 14 mls of EB into 7x2 ml fractions.</p>
Nucleotide Exchange and TEV cleavage: After elution the protein was concentrated to a final volume of 5 mls for nucleotide exchange and the protein concentration measured. To this was added a 25-fold excess of Gpp(NH)p, 5 mM EDTA, 1 mM DTT 100 microlitres of TEV protease and 14 microlitres of calf alkaline phosphatase (10,000U/ml), mixed and left overnight at 4°C.

The next day MgCl_2 was added to a final concentration of 10 mM before gel filtration.

GF buffer: 50 mM Tris pH 8.0, 500 mM NaCl, 0.5mM TCEP

Any remaining TEV protease and uncleaved RAC3 was removed by placing 200 μl of 50 % Ni-NTA agarose in a 1.5 ml eppendorf tubes, add 1ml of GF buffer mix, spin down and remove buffer. Repeat this resin wash step once.

Add the TEV treated protein sample to the resin and mix for 30 min. Finally spin down resin and collect the supernatant which contains the cleaved RAC3A.

Concentration : RAC3A was concentrated to 22 mg/ml before aliquoting into 50 microl volumes and freezing in the -80°C freezer.

Mass spec characterization :

Native Mass Spec for detection of non-covalently bound complexes indicated:

8+ charge state	exp mass	9+ charge state	exp mass	abs diff*	rel diff
A 2558.2	20 458	A 2274.0	20 457	+545	
B 2560.8	20 478	B 2276.4	20 479		+Na ⁺
C 2563.4	20 499	C 2278.4	20 497		+2Na ⁺

*rel. to 19 912

Peaks A/B/C correspond to RAC3A plus Na-GppNHp or Na-GTP (+545) with one or two additional Na ions.

Crystallisation: Crystals grew from a 1:2 ratio mix of RAC3A(GppNHp)-to-reservoir (0.1M BIS-TRIS pH 5.5; 25 % PEG₃₃₅₀).

Data Collection: Resolution: 1.9 Å; **X-ray source:** Rotating anode, Rigaku FR-E superbright