University of Dundee

Standard Operating Procedure

Preparation of active ΔPH PKBα S473D

Enzyme description:- Active Δ1-117 PKBα S473D

Source:- Recombinant

Expression system:- Baculovirus expression vector system (BEVS)/Insect cells

Tag:- His(6)

Purification method:- Ni^{2+}-NTA agarose.

Expression level:- 2-3 mg/L

Molecular mass:- 48 kDa by SDS-PAGE

Purity:- >80%

Contaminants:-
The preparation also contains a number of Ni^{2+}-NTA agarose binding proteins from insect
cells. The level of these contaminating proteins varies from preparation to preparation.

Activation protocol:-

Δ1-117 PKBα S473D (0.2 mg/ml – 4 μM) is activated in 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1 % β-mercaptoethanol, 10mM magnesium acetate, 0.1mM ATP with 3.3 μg/ml PDK1 for 30 min at 30°C. Following activation the preparation is made 400 mM NaCl by addition of 1/5 volume of 50 mM Tris/HCl pH 7.5, 2 M NaCl, 0.1 mM EGTA, 0.1 % β-mercaptoethanol and the PDK1 is removed by chromatography of the preparation on Heparin Sepharose (PDK1 binds very strongly to Heparin - PKB is in the flowthrough). The Δ1-117 PKBα S473D is then dialysed into enzyme storage buffer prior to storage at –80°C.

Enzyme storage buffer:-

50 mM Tris/HCl pH 7.5, 270 mM sucrose, 150 mM NaCl, 0.1 mM EGTA, 0.1 % β-mercaptoethanol, 0.2 mM PMSF,1 mM Benzamidine.

Storage temperature:- Aliquot, snap freeze and store at -70°C.

CLONE DATA SHEET - ΔPH-PKBα–S473D

Protein	Human PKBα Δ1–117 (ΔPH domain PKBα) S473D
Accession no	M63167, note the sequence differs to that of the database entry: S478 of the database entry is G389 of the fusion protein.
Tags	His(6)
Baculovirus-expressed protein	MSYYHHHHHHDYDIPTTENLYFQGAMGS**MDFRSGSPSDNS GAEEMEVSLAKPKHRVTMNEFEYLKLLGKGTFGKVILVKEK ATGRYYAMKILKKEVIVAKDEVAHTLTENRVLQNSRHPFLT ALKYSFQTHDRLCFVMEYANGGELFFHLSRERVFSEDRARF YGAEIVSALDYLHSEKNVVYRDLKLENLMLDKDGHIKITDF GLCKEGIKDGATMKTFCGTPEYLAPEVLEDNDYGRAVDW WGLGVVMYEMMCGRLPFYNQDHEKLFELILMEEIRFPRTLG PEAKSLLSGLLKKDPKQRLGGGSEDAKEIMQHRFFAGIVW QHVYEKKLSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQD DSMECVDSERRPHFPQFDYSASGTA**
Native sequence	Residue 29 of the His_6-tagged protein is equivalent to Met 118 of PKBα. S473 has been mutated to D (S384D in the fusion protein). There is a His(6)-tag at residues 5-10 of the fusion protein.
Protease cleavage site	ENLYFQ (rTEV protease) residues 18 – 23 of His_6-tagged protein
Cloning sites	A novel *Bam*HI site was introduced immediately 5′ to the initiator Met 118 by PCR. The resulting *Bam*HI/*Kpn*I fragment was sub-cloned into the *Bam*HI/*Kpn*I sites of pFastBAC HTb.
ORF of PKBα Δ1–117 S473D	ATGGACTTCCGGTCGGGCTCACCCAGTGACAACTCAGGGGCTGAAGAG ATGGAGGTGTCCCTGGCCAAGCCCAAGCACCGCGTGACCATGAACGAG TTTGAGTACCTGAAGCTGCTGGGCAAGGGCACTTTCGGCAAGGTGATC CTGGTGAAGGAGAAGGCCACAGGCCGCTACTACGCCATGAAGATCCTC AAGAAGGAAGTCATCGTGGCCAAGGACGAGGTGGCCCACACACTCACC GAGAACCGCGTCCTGCAGAACTCCAGGCACCCCTTCCTCACAGCCCTG AAGTACTCTTTCCAGACCCACGACCGCCTCTGCTTTGTCATGGAGTAC

GCCAACGGGGGCGAGCTGTTCTTCCACCTGTCCCGGGAGCGTGTGTTC
TCCGAGGACCGGGCCCGCTTCTATGGCGCTGAGATTGTGTCAGCCCTG
GACTACCTGCACTCGGAGAAGAACGTGGTGTACCGGGACCTCAAGCTG
GAGAACCTCATGCTGGACAAGGACGGGCACATTAAGATCACAGACTTC
GGGCTGTGCAAGGAGGGGATCAAGGACGGTGCCACCATGAAGACCTTT
TGCGGCACACCTGAGTACCTGGCCCCCGAGGTGCTGGAGGACAATGAC
TACGGCCGTGCAGTGGACTGGTGGGGGCTGGGCGTGGTCATGTACGAG
ATGATGTGCGGTCGCCTGCCCTTCTACAACCAGGACCATGAGAAGCTT
TTTGAGCTCATCCTCATGGAGGAGATCCGCTTCCCGCGCACGCTTGGT
CCCGAGGCCAAGTCCTTGCTTTCAGGGCTGCTCAAGAAGGACCCCAAG
CAGAGGCTTGGCGGGGGCTCCGAGGACGCCAAGGAGATCATGCAGCAT
CGCTTCTTTGCCGGTATCGTGTGGCAGCACGTGTACGAGAAGAAGCTC
AGCCCACCCTTCAAGCCCCAGGTCACGTCGGAGACTGACACCAGGTAT
TTTGATGAGGAGTTCACGGCCCAGATGATCACCATCACACCACCTGAC
CAAGATGACAGCATGGAGTGTGTGGACAGCGAGCGCAGGCCCCACTTC
CCCCAGTTCGACTACTCGGCCAGCGGCACGGCCtga