Purification and crystallisation of ALK2 (R206H) mutation with M4K Pharma compounds.

ALK2 (R206H) :

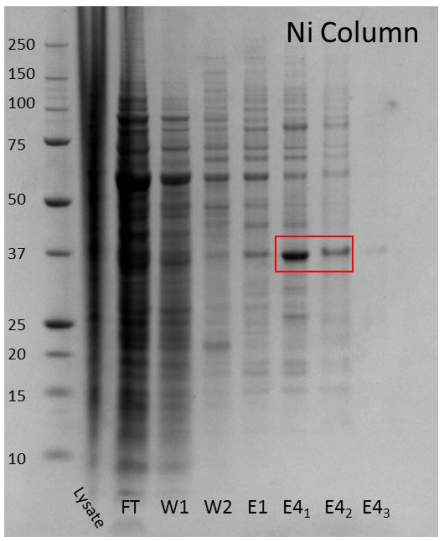
MGHHHHHHSSGVDLGTENLYFQ/*SMTTNVGDSTLADLLDHSCTSGSGSGLPFLVQRTVA**H**QITLLECVGKGRYG
EVWRGSWQGENVAVKIFSSRDEKSWFRETELYNTVMLRHENILGFIASDMTSRHSSTQLWLITHYHEMGSLYDYL
QLTTLDTVSCLRIVLSIASGLAHLHIEIFGTQGKPAIAHRDLKSKNILVKKNGQCCIADLGLAVMHSQSTNQLDVGNN
PRVGTKRYMAPEVLDETIQVDCFDSYKRVDIWAFGLVLWEVARRMVSNGIVEDYKPPFYDVVPNDPSFEDMRKV
VCVDQQRPNIPNRWFSDPTLTSLAKLMKECWYQNPSARLTALRIKKTLTKID

/* denotes Tev cleavage site

1L pellet from insect cell expression.

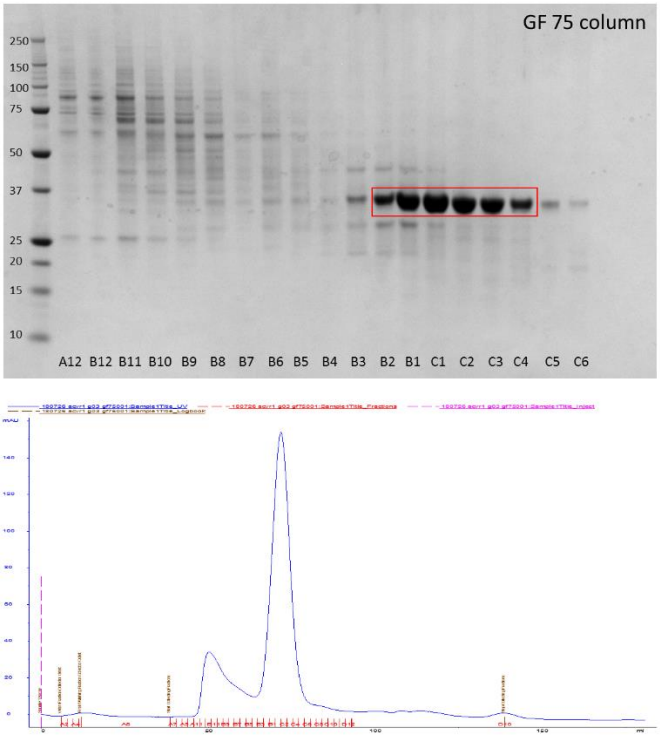
Purification:

- Thaw pellets in luke warm water
- Sonicate sample for 3 min (5 on, 10 off) on ice.
- Add sample to centrifuge tube and add 1ml PEI (5%) to precipitate DNA. (final concentration 0.15%)
- Spin at 21.5k rpm for 50 minutes.
- Incubate lysate with 2ml pre-equilibrated Ni-NTA beads at 4C for 1h. (500mM NaCl, 50mM HEPES, 5mM Imidazole, 5% glycerol, ph7.5)
- Spin down beads at 700g for 10 minutes to separate beads from lysate.
- Pour off lysate.
- Resuspend beads in 50ml binding buffer and load onto gravity flow column (collect flow through)
- Wash with 30ml wash buffer (500mM NaCl, 50mM HEPES, 30mM Imidazole, 5% glycerol, ph7.5).
- Elute in 7ml elution buffer 1 (500mM NaCl, 50mM HEPES, 50mM Imidazole, 5% glycerol, ph7.5).
- Elute in 3 x 7ml elution buffer 4 (500mM NaCl, 50mM HEPES, 250mM Imidazole, 5% glycerol, ph7.5).
- Add 1mM TCEP to all fractions.
- Run samples on an SDS PAGE gel (mix 5ul of loading dye with 15ul sample, boil for 3 minutes and load 10ul onto the gel.) and run at 160V for 50 minutes.
- Add Tev protease to fractions which contain protein ($E4_1$ and $E4_2$)
- Incubate at 4C overnight.



Samples corresponding to the nickle column fractions run on an SDS-PAGE (170V for 50 min). Firstly initial lysate after centrifugation, then FT = flow through after binding to beads, W1 = wash 1, W2 = wash 2, E1 = elution with E1 elution buffer, E4$_1$ – E4$_3$ = three elutions with E4 elution buffer.

- Concentrate down boxed samples to <5ml and run on a pre-equilibrated GF 75 column using standard gel filtration buffer (300mM NaCl, 50mM HEPES, ph7.5).
- Collect fractions and run a gel of peak.



ALK2 SDS-PAGE gel (top) shows the samples from the relevant size exclusion column fractions, corresponding to the UV peak (bottom). The boxed fractions are the samples taken forward for crystallisation.

Crystallisation:

- Concentration of protein measured and mixed with a 1:1 ratio of FKBP12 previously purified.
- Sample concentrated down to 8.4mg/ml.
- Sample divided into 3 and 0.5mM compound added to each tube. (Compounds used M4K2009, M4K3003, M4K3007)
- Sample spun at 13000rpm on a benchtop centrifuge for 10 minutes.

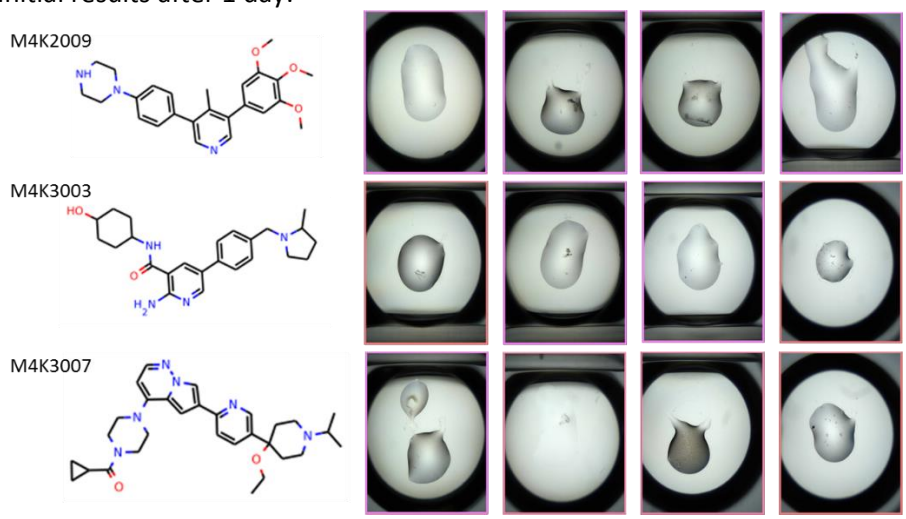
ACVR1Z-c082, e086 p012 R206H long form.
FKBP1AA-c002 (GST-tagged) p018

SGC ID: XX06ACVR1Z-p001

Protein complex concentration 8.4 mg/ml
Ligand concentration at 0.5mM
Single sized drops (150nL), 3 drop ratio (1:2, 1:1, 2:1)

Bar code	Screen	Additives	Incubated temp:
CI071100	JCSG7	M4K3007	20C
CI071229	LFS6	M4K3007	20C
CI071299	HCS3	M4K3007	4C
CI071298	HIN3	M4K3007	4C
CI071297	JCSG7	M4K3003	20C
CI071296	LFS6	M4K3003	20C
CI071295	HCS3	M4K3003	4C
CI071294	HIN3	M4K3003	4C
CI071293	JCSG7	M4K2009	20C
CI071292	LFS6	M4K2009	20C
CI071291	HCS3	M4K2009	4C
CI071290	HIN3	M4K2009	4C

Initial results after 1 day:



Drops containing crystalline matter of the complex ALK2/FKBP12 with the corresponding compound on the left.