Monolith X Protocol MOX-P-103

Cereblon E3 Ligase – SD-36 (PROTAC) – STAT3

A proteolysis targeting chimera (PROTAC) is a heterobifunctional small molecule composed of two active domains and a linker, capable of removing specific unwanted proteins. Rather than acting as a conventional enzyme inhibitor, a PROTAC works by inducing selective intracellular proteolysis. PROTACs consist of two covalently linked protein-binding molecules: one capable of engaging an E3 ubiquitin ligase, and another one that binds to a target protein meant for degradation. Cereblon forms an E3 ubiquitin ligase complex with damaged DNA binding protein 1 (DDB1), which then can ubiquitinate other proteins. Signal transducer and activator of transcription 3 (STAT3) is a transcription factor. SD-36 is a potent and efficacious STAT3 PROTAC degrader. It is composed of the STAT3 inhibitor SI-109, a linker, and an analog of Cereblon ligand Lenalidomide for E3 ubiquitin ligase.

protein – small molecule | PROTACs | ternary complex | Hook effect

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A1. Target/Fluorescent Molecule

Cereblon E3 ligase complex (CRBN/DDB1)

CRBN
uniprot.org/uniprot/Q96SW2

DDB1
uniprot.org/uniprot/Q16531

A2. Molecule Class/Organism

E3 protein ligase complex
Homo sapiens (Human)

A3. Sequence/Formula

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MGSSHHHHHH SSGENLYFQG SMEAKKPNII NFDTSLPTSH TYLGADMEEF HGRTLHDDDS CQVIPVLPQV MMILIPGQTL
PLQLFHPQEV SMVRNLIQKD RTFAVLAYSN VQEREAQFGT TAEIYAYREE QDFGIEIVKV KAIGRQRFKV LELRTQSDGI
QQAKVQILPE CVLPSTMSAV QLESLNKCQI FPSKPVSRED QCSYKWWQKY QKRKFHCANL TSWPRWLYSL YDAETLMDRI
KKQLREWDEN LKDDSLPSNP IDFSYRVAAC LPIDDVLRIQ LLKIGSAIQR LRCELDIMNK CTSLCCKQCQ ETEITTKNEI
FSLSLCGPMA AYVNPHGYVH ETLTVYKACN LNLIGRPSTE HSWFPGYAWT VAQCKICASH IGWKFTATKK DMSPQKFWGL
TRSALLPTIP DTEDEISPDK VILCL
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MSYNYVVTAQ KPTAVNGCVT GHFTSAEDLN LLIAKNTRLE IYVVTAEGLR PVKEVGMYGK IAVMELFRPK GESKDLLFIL
TAKYNACILE YKQSGESIDI ITRAHGNVQD RIGRPSETGI IGIIDPECRM IGLRLYDGLF KVIPLDRDNK ELKAFNIRLE
ELHVIDVKFL YGCQAPTICF VYQDPQGRHV KTYEVSLREK EFNKGPWKQE NVEAEASMVI AVPEPFGGAI IIGQESITYH
NGDKYLAIAP PIIKQSTIVC HNRVDPNGSR YLLGDMEGRL FMLLLEKEEQ MDGTVTLKDL RVELLGETSI AECLTYLDNG
VVFVGSRLGD SQLVKLNVDS NEQGSYVVAM ETFTNLGPIV DMCVVDLERQ GQGQLVTCSG AFKEGSLRII RNGIGIHEHA
SIDLPGIKGL WPLRSDPNRE TDDTLVLSFV GQTRVLMLNG EEVEETELMG FVDDQQTFFC GNVAHQQLIQ ITSASVRLVS
QEPKALVSEW KEPQAKNISV ASCNSSQVVV AVGRALYYLQ IHPQELRQIS HTEMEHEVAC LDITPLGDSN GLSPLCAIGL
WTDISARILK LPSFELLHKE MLGGEIIPRS ILMTTFESSH YLLCALGDGA LFYFGLNIET GLLSDRKKVT LGTQPTVLRT
FRSLSTTNVF ACSDRPTVIY SSNHKLVFSN VNLKEVNYMC PLNSDGYPDS LALANNSTLT IGTIDEIQKL HIRTVPLYES
PRKICYQEVS QCFGVLSSRI EVQDTSGGTT ALRPSASTQA LSSSVSSSKL FSSSTAPHET SFGEEVEVHN LLIIDQHTFE
VLHAHQFLQN EYALSLVSCK LGKDPNTYFI VGTAMVYPEE AEPKQGRIVV FQYSDGKLQT VAEKEVKGAV YSMVEFNGKL
LASINSTVRL YEWTTEKELR TECNHYNNIM ALYLKTKGDF ILVGDLMRSV LLLAYKPMEG NFEEIARDFN PNWMSAVEIL
DDDNFLGAEN AFNLFVCQKD SAATTDEERQ HLQEVGLFHL GEFVNVFCHG SLVMQNLGET STPTQGSVLF GTVNGMIGLV
TSLSESWYNL LLDMQNRLNK VIKSVGKIEH SFWRSFHTER KTEPATGFID GDLIESFLDI SRPKMQEVVA NLQYDDGSGM
KREATADDLI KVVEELTRIH
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A4. Purification Strategy/Source

Crelux GmbH
Construct ID: DLS7/CXU4, Lot-ID: PH15311

A5. Stock Concentration/Stock Buffer

6.55 mg/mL | 37.2 µM
10 mM HEPES pH 7.0, 240 mM NaCl, 1 mM TCEP

A6. Molecular Weight/Extinction Coefficient

48.8 kDa (CRBN), 127.0 kDa (DDB1)
166,510 $M^{-1}cm^{-1}$ (ε_{280})

A7. Dilution Buffer

20 mM HEPES pH 7.4, 150 mM NaCl, 0.5 mM TCEP, 0.01 % Pluronic® F-127

A8. Labeling Strategy

Monolith Protein Labeling Kit RED – NHS 2nd Generation (MO-L011, NanoTemper Technologies GmbH)
1* Dye RED-NHS 2nd Generation (10 µg) | 1* B-Column

A9. Labeling Procedure

1. Mix 5 µL of 37.2 µM µM CRBN/DDB1 with 15 µL of dilution buffer to obtain 20 µL of ~10 µM CRBN/DDB1.
2. Add 25 µL of DMSO to Dye RED-NHS 2nd Generation (10 µg) to obtain a ~600 µM solution. Mix the dye thoroughly by vortexing and make sure that all dye is dissolved.
3. Mix 1 µL of the 600 µM dye solution with 19 µL of dilution buffer to obtain 20 µL of a 30 µM dye solution (3x protein concentration).
4. Mix CRBN/DDB1 and dye in a 1:1 volume ratio (40 µL final volume, 2.5% final DMSO concentration).
5. Incubate for 30 minutes at room temperature in the dark.
6. In the meantime, remove the top cap of the B-Column and pour off the storage solution. Remove the bottom cap and place with adapter in a 15 mL tube.
7. Fill the column with dilution buffer and allow it to enter the packed resin bed completely by gravity flow. Discard the flow through collected. Repeat this step 3 more times.
8. Add 40 µL of the labeling reaction from step 4 to the center of the column and let sample enter the resin bed completely.
9. Add 550 µL of dilution buffer after the sample has entered and discard the flow through.
10. Place column in a new collection tube, add 400 µL of dilution buffer and collect the eluate.
11. Prepare 8 µL aliquots of the labeled CRBN/DDB1 (~0.5 µM) and immediately store at -80°C.

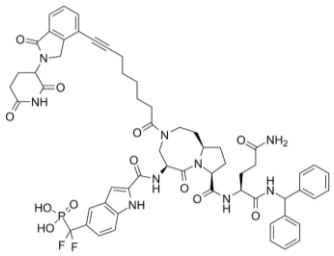
A10. Labeling Efficiency

Measurement of protein concentration and degree of labeling (DOL) using a NanoDrop™:
nanotempertech.com/dol-calculator

Absorbance A_{280}	0.100	Protein concentration	0.57 µM
Absorbance A_{650}	0.118	Degree-of-labeling (DOL)	1.06

B1. Ligand/Non-Fluorescent Binding Partner

SD-36



Signal transducer and activator of transcription 3 (STAT3)
uniprot.org/uniprot/P40763

B2. Molecule Class/Organism

PROTAC

Transcription factor
Homo sapiens (Human)

B3. Sequence/Formula

$C_{59}H_{62}F_2N_9O_{12}P$

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GQANHPTAAV VTEKQQMLEQ HLQDVRKRVQ DLEQKMKVVE NLQDDFDFNY
KTLKSQGDMQ DLNGNNQSVT RQKMQQLEQM LTALDQMRRS IVSELAGLLS
AMEYVQKTLT DEELADWKRR QQIACIGGPP NICLDRLENW ITSLAESQLQ
TRQQIKKLEE LQQKVSYKGD PIVQHRPMLE ERIVELFRNL MKSAFVVERQ
PCMPMHPDRP LVIKTGVQFT TKVRLLVKFP ELNYQLKIKV CIDKDSGDVA
ALRGSRKFNI LGTNTKVMNM EESNNGSLSA EFKHLTLREQ RCGNGGRANC
DASLIVTEEL HLITFETEVY HQGLKIDLET HSLPVVVISN ICQMPNAWAS
ILWYNMLTNN PKNVNFFTKP PIGTWDQVAE VLSWQFSSTT KRGLSIEQLT
TLAEKLLGPG VNYSGCQITW AKFCKENMAG KGFSFWVWLD NIIDLVKKYI
LALWNEGYIM GFISKERERA ILSTKPPGTF LLRFSESSKE GGVTFTWVEK
DISGKTQIQS VEPYTKQQLN NMSFAEIIMG YKIMDATNIL VSPLVYLYPD
IPKEEAFGKY CRPESQEHPE ADPGSAAPYL KTKFICVTPG SGENLYFQGH HHHHH
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B4. Purification Strategy/Source

SD-36
MedChemExpress
HY-129602

Recombinant
Crelux GmbH

B5. Stock Concentration/Stock Buffer

11.6 mg/mL | 10 mM
DMSO

12.87 mg/mL | 195 µM
100 mM Tris-HCl pH 8.5, 100 mM NaCl, 2 mM DTT

B6. Molecular Weight/Extinction Coefficient

1158.15 Da	66.1 kDa 84,340 $M^{-1}cm^{-1}$ (ε_{280})

B7. Serial Dilution Preparation

1. Dissolve 5 mg of SD-36 in 432 µL of DMSO to obtain a 10 mM stock solution.
2. Mix 2 µL of 10 mM SD-36 with 98 µL of DMSO to obtain 100 µL of a 200 µM SD-36 solution.
3. Mix 4 µL of 200 µM SD-36 with 196 µL of dilution buffer to obtain 100 µL of a 4 µM SD-36 solution.
4. Mix 20 µL of DMSO with 980 µL of dilution buffer to obtain 1 mL of dilution buffer with 2% DMSO.
5. Mix 4 µL of labeled CRBN/DDB1 (~0.5 µM) with 96 µL of dilution buffer to obtain 200 µL of 20 nM CRBN/DDB1.
6. Mix 1 µL of 195 µM STAT3 with 121 µL of dilution buffer to obtain 122 µL of 1.6 µM STAT3.

CRBN – SD-36 (binary complex)

7. Mix 100 µL of 20 nM CRBN with 100 µL of dilution buffer to obtain 200 µL of 10 nM CRBN.
8. Take a fresh 0.5 mL tube and mix 160 µL of 10 nM CRBN with 160 µL of dilution buffer containing 2% DMSO to obtain 320 µL of a 5 nM CRBN solution.
9. Prepare a PCR-rack with 16 PCR tubes. Transfer 20 µL of the 5 nM CRBN solution into tubes **2** to **16**. Then, mix 20 µL of the 10 nM CRBN solution with 20 µL of the 4 µM SD-36 solution in tube **1**.
10. Prepare a 1:1 serial dilution by transferring 20 µL from tube to tube. Mix carefully by pipetting up and down.
11. Incubate for 5 minutes at room temperature in the dark before loading capillaries.

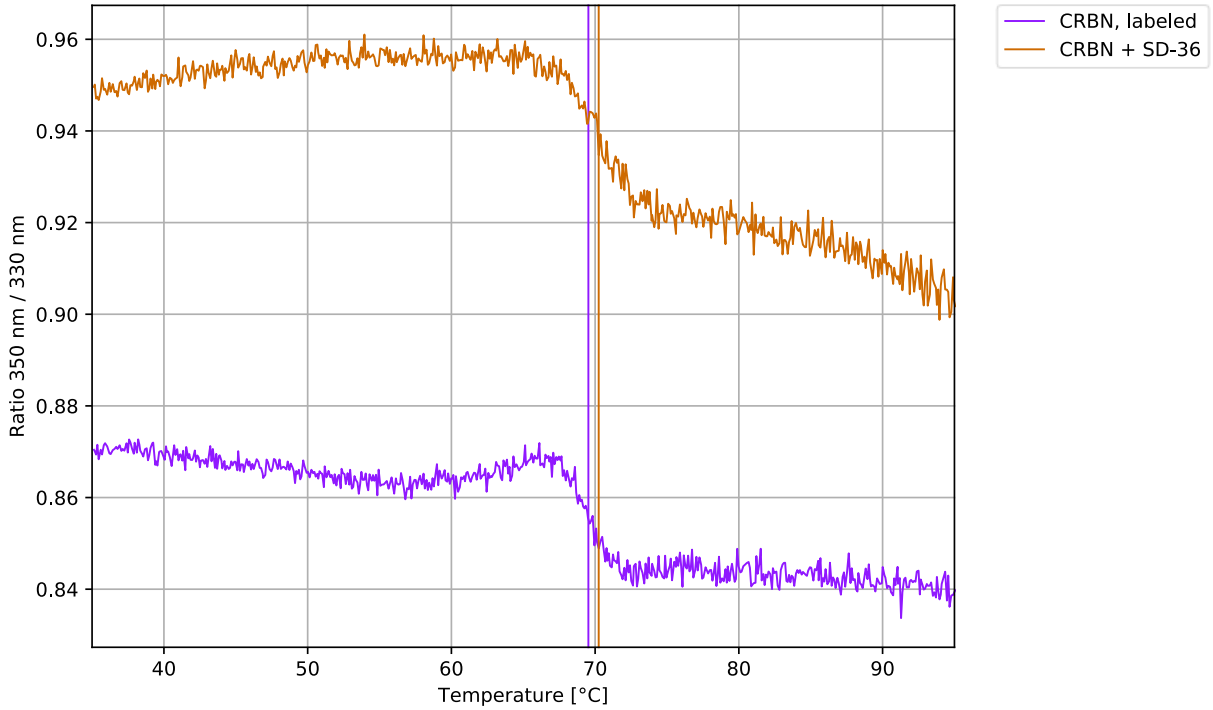
CRBB & STAT3 – SD-36 (ternary complex)

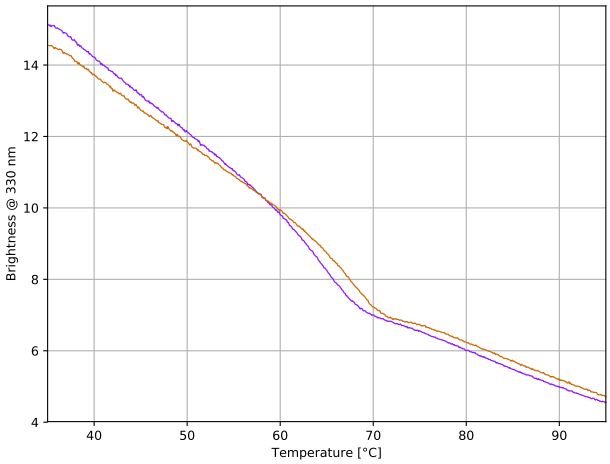
12. Mix 100 µL of 20 nM CRBN with 100 µL of 1.6 µM STAT3 to obtain 200 µL of 10 nM CRBN, 800 nM STAT3.
13. Take a fresh 0.5 mL tube and mix 160 µL of 10 nM CRBN, 800 nM STAT3with 160 µL of dilution buffer containing 2% DMSO to obtain 320 µL of a 5 nM CRBN, 400 nM STAT3solution.
14. Prepare a PCR-rack with 20 PCR tubes. Transfer 20 µL of the 5 nM CRBN, 400 nM STAT3solution into tubes **2** to **16**. Then, mix 20 µL of 4 µM SD-36 with 20 µL of 10 nM CRBN, 800 nM STAT3 in tube **1**.
15. Prepare a 1:1 serial dilution by transferring 20 µL from tube to tube. Mix carefully by pipetting up and down.
16. Incubate for 5 minutes at room temperature in the dark before loading capillaries.

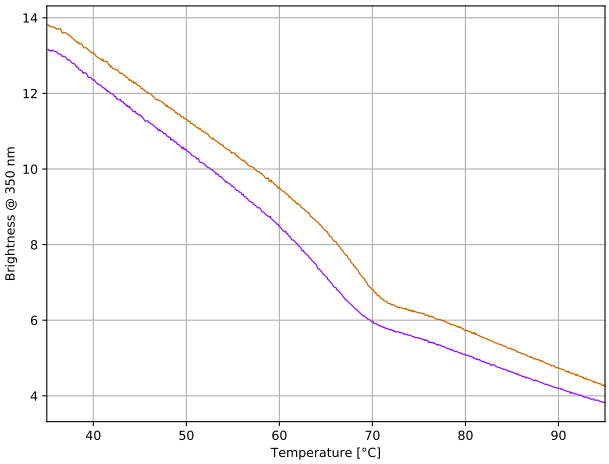
C. Tycho

Validation of structural integrity and functionality of labeled CRBN-DDB1 using Tycho NT.6:
nanotempertech.com/tycho

CRBN, labeled	8 µL of B-Column eluate (~0.5 µM) + 2 µL of dilution buffer with 2% DMSO	T_i = 69.5°C
CRBN + SD-36	8 µL of B-Column eluate (~0.5 µM) + 2 µL of 4 µM SD-36	T_i = 70.2°C

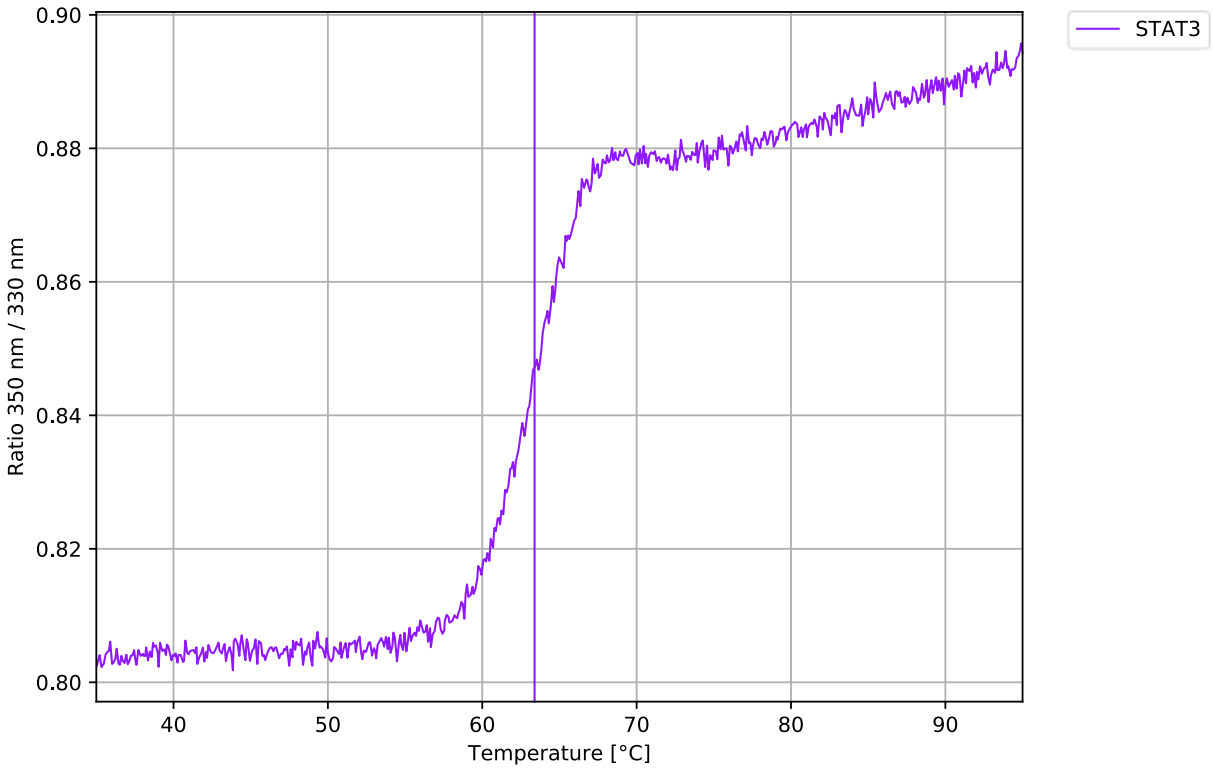


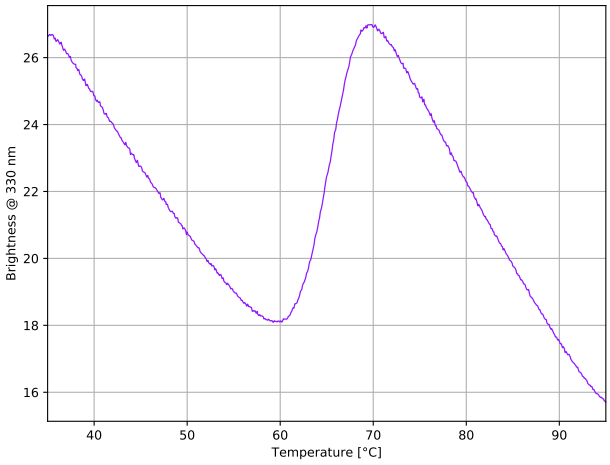


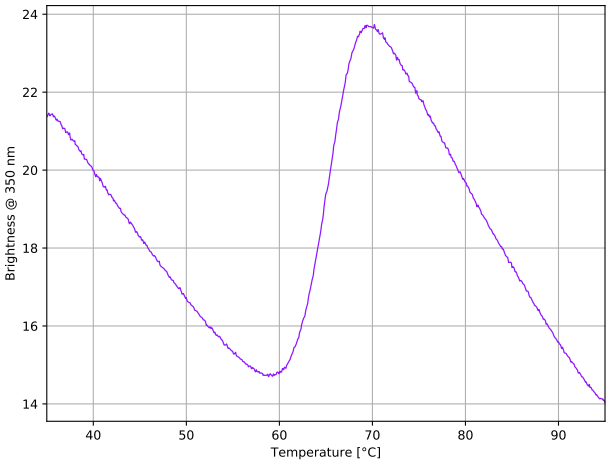


Validation of structural integrity of STAT3 using Tycho NT.6:
nanotempertech.com/tycho

STAT3	10 µL of 1.6 µM STAT3	T_i = 63.4°C







D1. Monolith System/Capillaries

Monolith X (NanoTemper Technologies GmbH)
Monolith Premium Capillaries (MO-K025, NanoTemper Technologies GmbH)

D2. Monolith Software

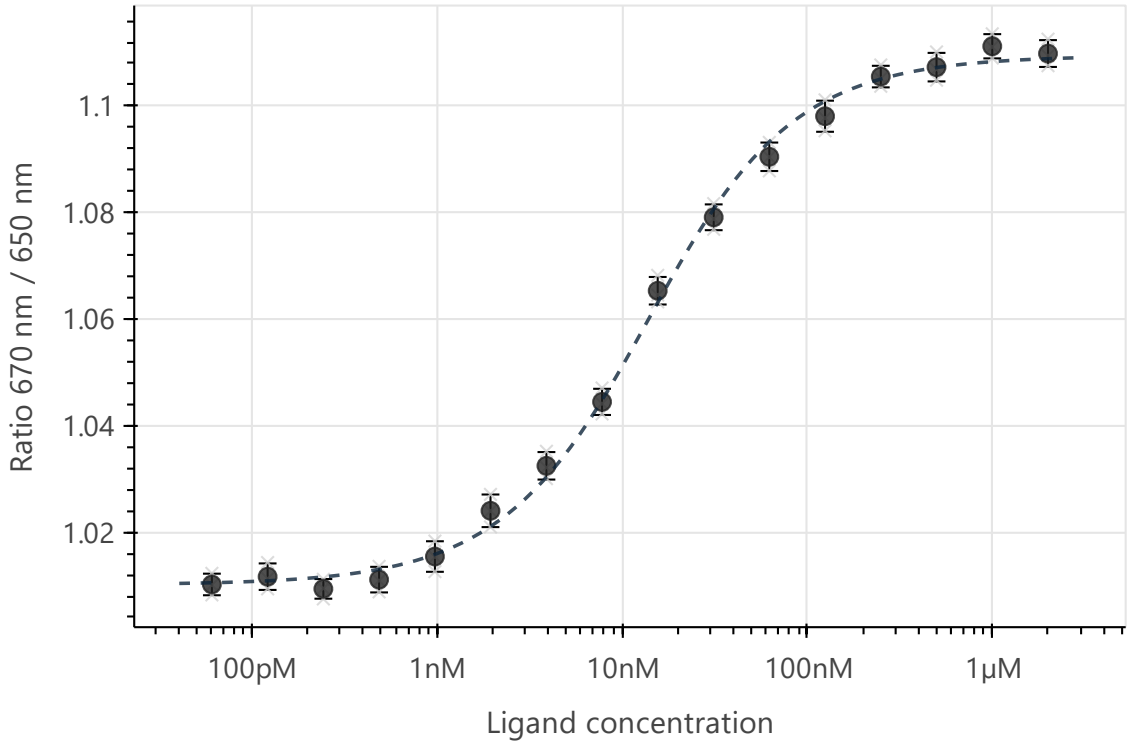
MO.Control v2.4.2 (NanoTemper Technologies GmbH)
nanotempertech.com/monolith-mo-control-software

D3. Monolith Experiment (Assay Buffer/Concentrations/Temperature/Excitation Power)

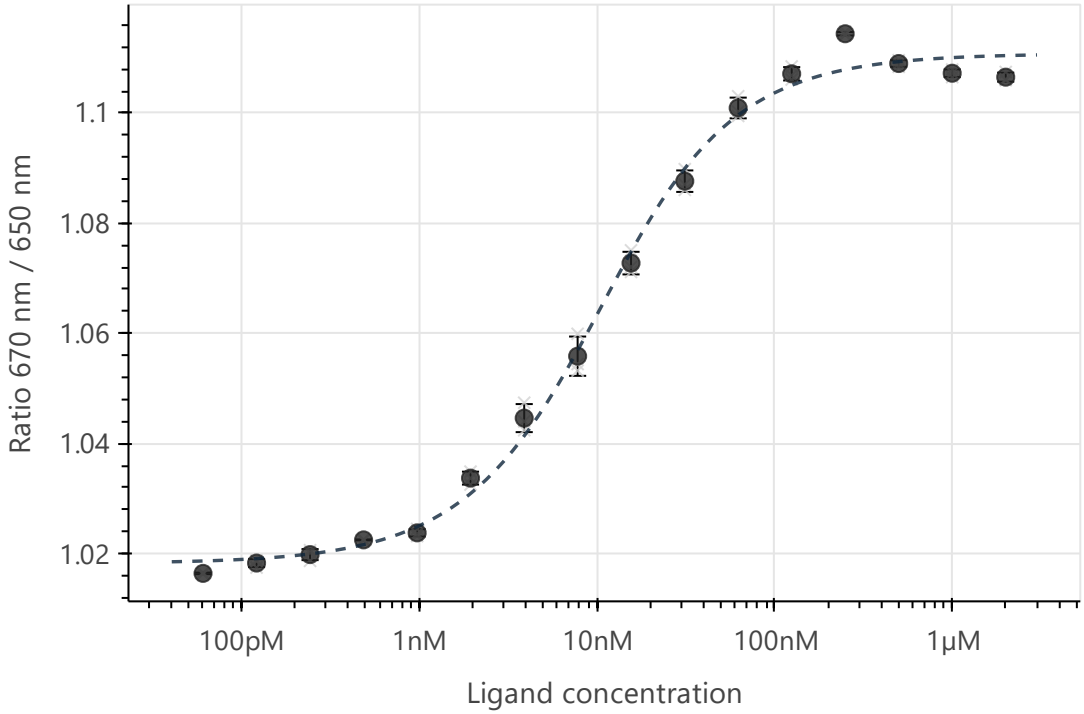
20 mM HEPES pH 7.4, 150 mM NaCl, 0.5 mM TCEP, 0.01 % Pluronic® F-127, 1% DMSO
5 nM CRBN-DDB1 | 2 µM – 61 pM SD-36 | 20°C | 100% Excitation Power

D4. Monolith Results (Capillary Scan/Time Traces/Dose Response)

CRBN – SD-36 (binary complex) | K_d = 11.3 ± 0.8 nM (S/N = 51.2)

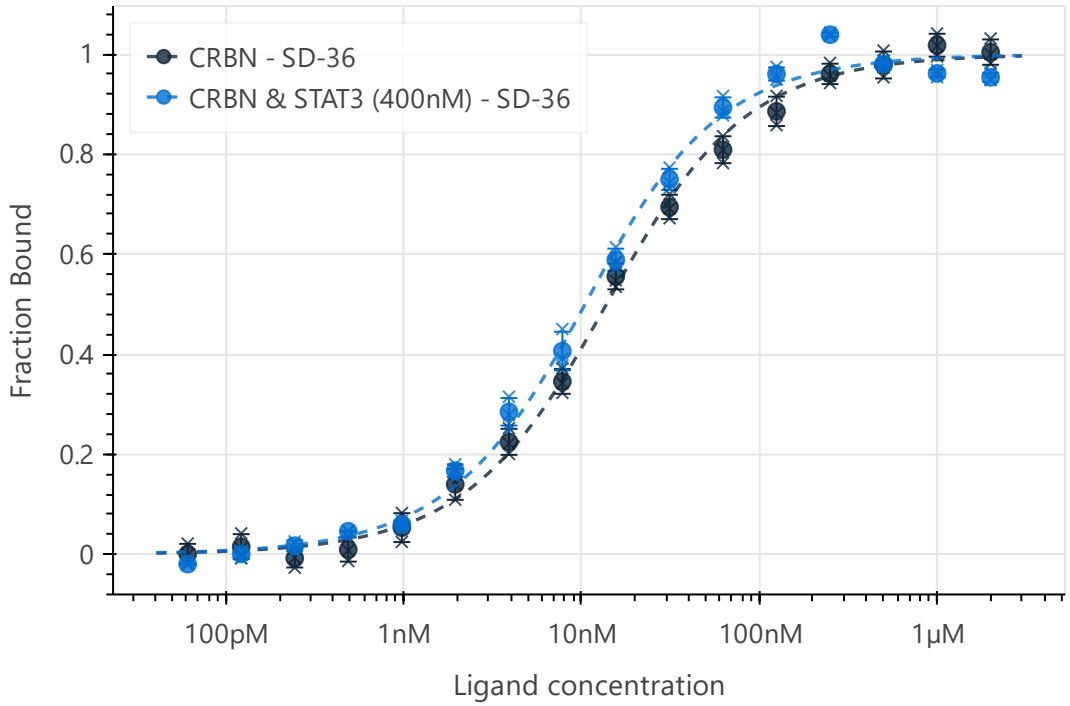


CRBN & STAT3 – SD-36 (ternary complex) | K_d = 7.98 ± 0.87 nM (S/N = 34.3)



Cooperativity factor: $\alpha = \frac{11.3\ \text{nM}}{7.98\ \text{nM}} \approx 1.4$

Overlay



D5. Reference Results/Supporting Results

N/A

E. Contributors

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