Monolith X Protocol MOX-P-104

STAT3 – SD-36 (PROTAC) – Cereblon

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

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

A2. Molecule Class/Organism

Transcription factor
Homo sapiens (Human)

A3. Sequence/Formula

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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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A4. Purification Strategy/Source

Recombinant
Crelux GmbH
Construct ID: DDO3, Lot-ID: PC10912

A5. Stock Concentration/Stock Buffer

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

A6. Molecular Weight/Extinction Coefficient

66.1 kDa
84,340 $M^{-1}cm^{-1}$ (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-L014, NanoTemper Technologies GmbH)
1* Dye RED-NHS 2nd Generation (10 µg) | 1* A-Column | 1* B-Column

A9. Labeling Procedure

1. Mix 2 µL of 195 µM µM STAT3 with 38 µL of dilution buffer to obtain 40 µL of ~10 µM STAT3.
2. Use the A-Column to perform a buffer exchange into dilution buffer.
   a. Invert A-Column to suspend slurry and twist off bottom (twist slightly in both directions).
   b. Loosen the cap of the column and place it in a 2 mL microcentrifuge collection tube.
   c. Centrifuge at **1500 × g** for **1 min** to remove excess liquid.
   d. Add 300 µL of dilution buffer and centrifuge at **1500 × g** for **1 min** (3x).
   e. Place 40 µL of the 10 µM STAT3 solution in the center of the resin.
   f. Place the sample in a **new** microcentrifuge collection tube and centrifuge at **1500 × g** for **2 min**.

   The collected flow-through should yield around 40 µL of ~5 µM STAT3 (~50% recovery).
3. 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.
4. Mix 2 µL of the 600 µM dye solution with 38 µL of dilution buffer to obtain 40 µL of a 30 µM dye solution (6x protein concentration).
5. Mix STAT3 and dye in a 1:1 volume ratio (80 µL final volume, 2.5% final DMSO concentration).
6. Incubate for 30 minutes at room temperature in the dark.
7. 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.
8. 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.
9. Add 80 µL of the labeling reaction from step 5 to the center of the column and let sample enter the resin bed completely.
10. Add 500 µL of dilution buffer after the sample has entered and discard the flow through.
11. Place column in a new collection tube, add 400 µL of dilution buffer and collect the eluate.
12. Prepare 8 µL aliquots of the labeled STAT3 (~0.5 µM) and immediately store at -80°C.

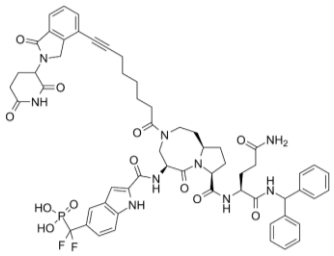
A10. Labeling Efficiency

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

Absorbance A_{280}	0.059	Protein concentration	0.66 µM
Absorbance A_{650}	0.086	Degree-of-labeling (DOL)	0.67

B1. Ligand/Non-Fluorescent Binding Partner

SD-36



Cereblon E3 ligase complex (CRBN/DDB1)

CRBN	DDB1
uniprot.org/uniprot/Q96SW2	uniprot.org/uniprot/Q16531

B2. Molecule Class/Organism

PROTAC

E3 protein ligase complex
Homo sapiens (Human)

B3. Sequence/Formula

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

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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

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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B4. Purification Strategy/Source

SD-36 MedChemExpress HY-129602	Crelux GmbH Construct ID: DLS7/CXU4, Lot-ID: PH15311

B5. Stock Concentration/Stock Buffer

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

B6. Molecular Weight/Extinction Coefficient

1158.15 Da	48.8 kDa (CRBN), 127.0 kDa (DDB1) 166,510 $M^{-1}cm^{-1}$ (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 20 µL of labeled STAT3 (~0.5 µM) with 480 µL of dilution buffer to obtain 500 µL of 20 nM STAT3.
6. Centrifuge at 15,000 rpm and 4°C for 30 minutes to remove aggregates. Then, carefully transfer the supernatant into a fresh tube and mix well by pipetting.
7. Mix 5.9 µL of 37.2 µM CRBN with 104.1 µL of dilution buffer to obtain 110 µL of 2 µM CRBN.

STAT3 – SD-36 (binary complex)

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

STAT3 & high concentration of CRBN – SD-36 (ternary complex, saturation)

13. Mix 100 µL of 20 nM STAT3 with 100 µL of 2 µM CRBN to obtain 200 µL of 10 nM STAT3, 1 µM CRBN.
14. Take a fresh 0.5 mL tube and mix 160 µL of 10 nM STAT3, 1 µM CRBN with 160 µL of dilution buffer containing 2% DMSO to obtain 320 µL of a 5 nM STAT3, 500 nM CRBN solution.
15. Prepare a PCR-rack with 20 PCR tubes. Transfer 20 µL of the 5 nM STAT3, 500 nM CRBN solution into tubes **2** to **16**. Then, mix 20 µL of 4 µM SD-36 with 20 µL of 10 nM STAT3, 1 µM CRBN in tube **1**.
16. Prepare a 1:1 serial dilution by transferring 20 µL from tube to tube. Mix carefully by pipetting up and down.
17. Incubate for 5 minutes at room temperature in the dark before loading capillaries.

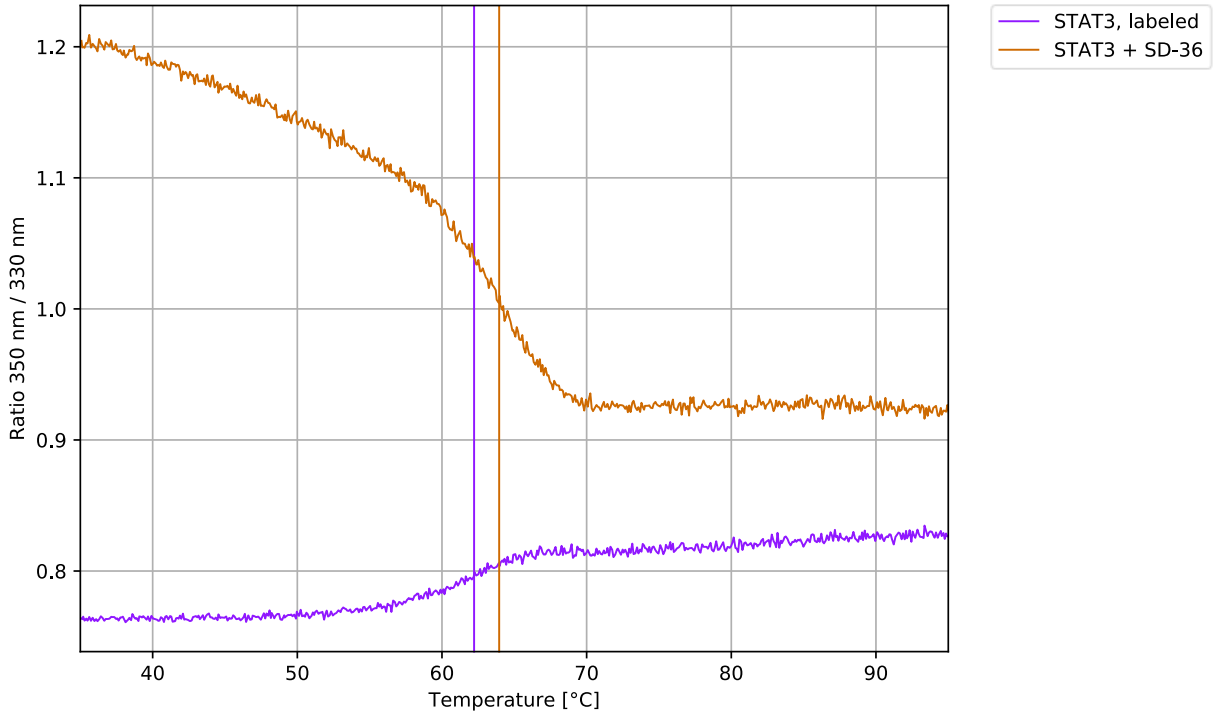
STAT3 & low concentration of CRBN – SD-36 (ternary complex, Hook effect)

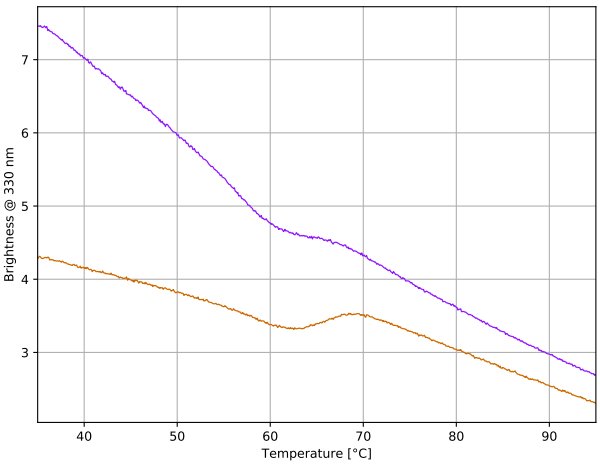
18. Mix 100 µL of 20 nM STAT3 with 8 µL of 2 µM CRBN and 92 µL of dilution buffer to obtain 200 µL of 10 nM STAT3, 80 nM CRBN.
19. Take a fresh 0.5 mL tube and mix 160 µL of 10 nM STAT3, 80 nM CRBN with 160 µL of dilution buffer containing 2% DMSO to obtain 320 µL of a 5 nM STAT3, 40 nM CRBN solution.
20. Prepare a PCR-rack with 20 PCR tubes. Transfer 20 µL of the 5 nM STAT3, 40 nM CRBN solution into tubes **2** to **16**. Then, mix 20 µL of 4 µM SD-36 with 20 µL of 10 nM STAT3, 80 nM CRBN in tube **1**.
21. Prepare a 1:1 serial dilution by transferring 20 µL from tube to tube. Mix carefully by pipetting up and down.
22. Incubate for 5 minutes at room temperature in the dark before loading capillaries.

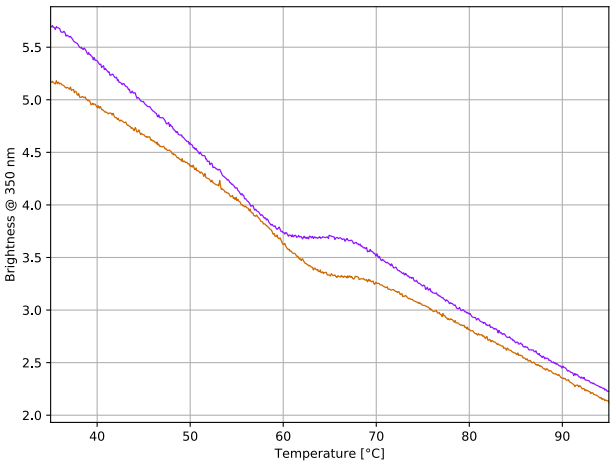
C. Tycho

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

STAT3, labeled	8 µL of B-Column eluate (~0.5 µM) + 2 µL of dilution buffer with 2% DMSO	T_i = 62.2°C
STAT3 + SD-36	8 µL of B-Column eluate (~0.5 µM) + 2 µL of 4 µM SD-36	T_i = 64.0°C







D1. Monolith System/Capillaries

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

D2. Monolith Software

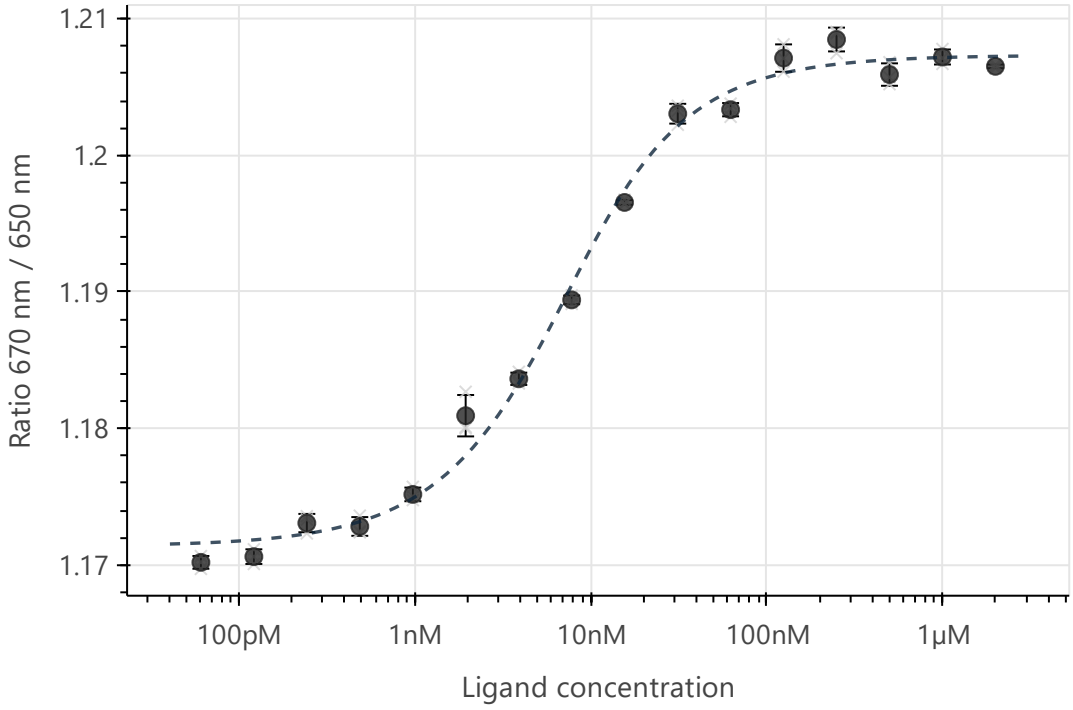
MO.Control v2.4.2 (NanoTemper Technologies GmbH)
nanotempertech.com/monolith-mo-control-software

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

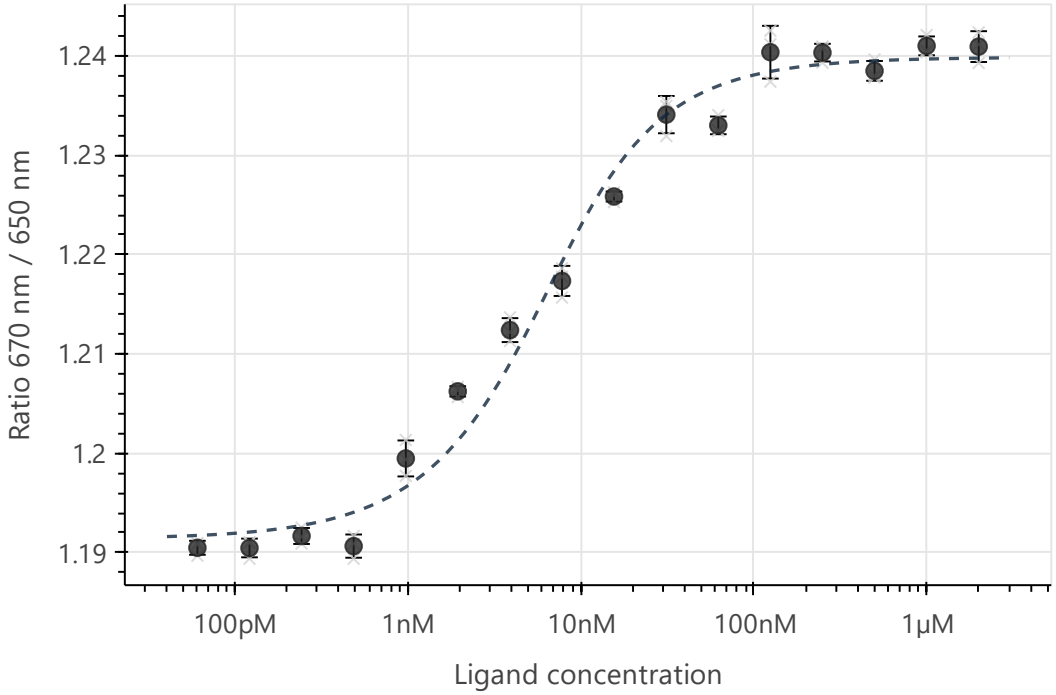
20 mM HEPES pH 7.4, 150 mM NaCl, 0.5 mM TCEP, 0.01 % Pluronic® F-127, 1% DMSO
5 nM STAT3 | 2 µM – 61 pM SD-36 | 20°C | 100% Excitation Power

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

STAT3 – SD-36 (binary complex) | K_d = 4.54 ± 0.65 nM (S/N = 28.5)

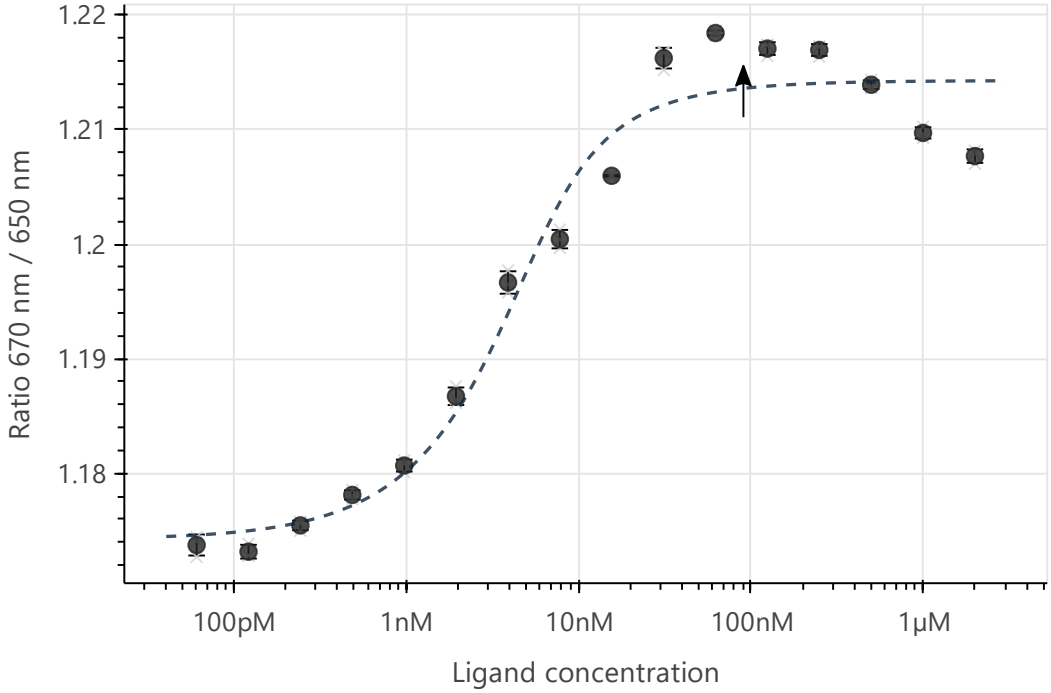


STAT3 & CRBN (500 nM) – SD-36 (ternary complex, saturation) | K_d = 3.64 ± 0.82 nM (S/N = 19.1)

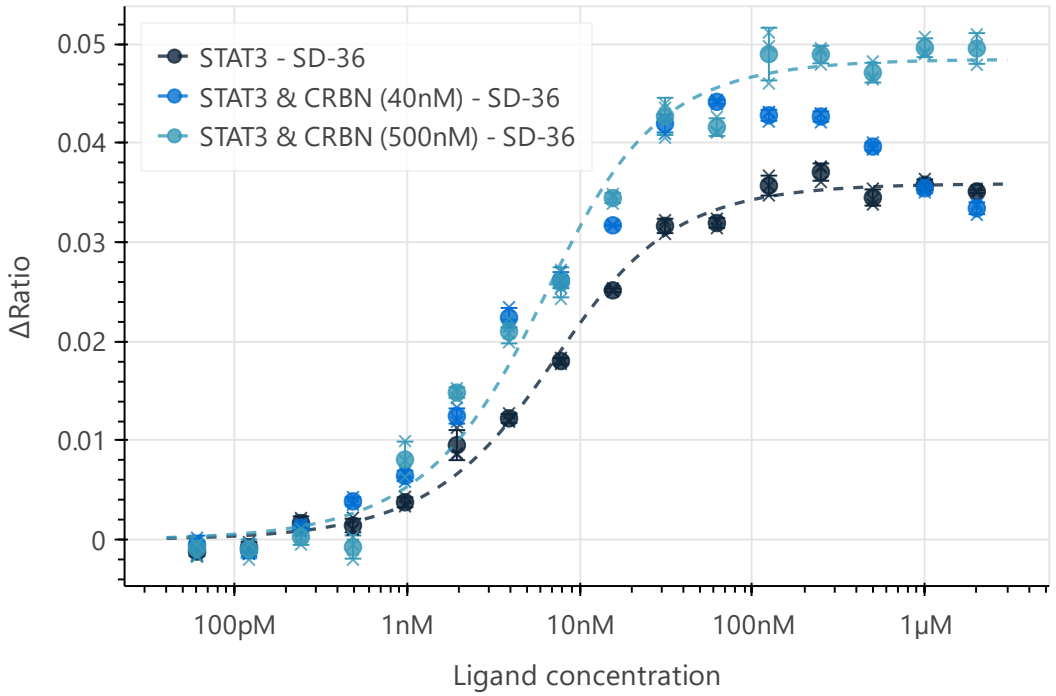


Cooperativity factor: $\alpha = \frac{4.54\ \text{nM}}{3.64\ \text{nM}} \approx 1.2$

STAT3 & CRBN (40 nM) – SD-36 (ternary complex, Hook effect)



Overlay



D5. Reference Results/Supporting Results

STAT3 – SD-36	K_d = 44.4 nM	Biolayer Interferometry (BLI)
	K_i = 9 nM	FP assay
		Bai et al, Cancer Cell 36, 498–511 (2019)

E. Contributors

Andreas Langer[1]

[1] NanoTemper Technologies GmbH, München, Germany | nanotempertech.com