Monolith X Protocol MOX-P-101

$BRD2^{BD2}$ – MZ1 (PROTAC) – VCB (VHL) E3 Ligase

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. The von Hippel-Lindau E3 Ligase (VHL) and the elongin BC proteins form together the functional VCB complex which is able to use the PROTAC MZ1 to bind to the bromodomain of BRD2.

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

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A1. Target/Fluorescent Molecule

$BRD2^{BD2}$
uniprot.org/uniprot/P25440

A2. Molecule Class/Organism

Bromo-domain containing protein

A3. Sequence/Formula

MHHHHHHSGV DLGTENLYFQ SMGKLSEQLK HCNGILKELL SKKHAAYAWP FYKPVDASAL GLHDYHDIIK HPMDLSTVKR KMENRDYRDA QEFAADVRLM FSNCYKYNPP DHDVVAMARK LQDVFEFRYA KMPD

A4. Purification Strategy/Source

Crelux GmbH
Construct ID: CRN1, Lot-ID: PC16670

A5. Stock Concentration/Stock Buffer

5.72 mg/mL | 362 µM
25 mM HEPES pH 7.5, 100 mM NaCl, 5% glycerol, 1 mM DTT

A6. Molecular Weight/Extinction Coefficient

15.8 kDa
17,420 $M^{-1}cm^{-1}$ (ε_{280})

A7. Dilution Buffer

50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM GSH, 0.01 % Pluronic® F-127

A8. Labeling Strategy

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

A9. Labeling Procedure

1. Prepare 50 µL of 25 µM $BRD2^{BD2}$ by mixing 3.45 µL of 362 µM $BRD2^{BD2}$ with 46.5 µL of Labeling Buffer NHS.
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 5 µL of the 600 µM dye solution with 45 µL of Labeling Buffer NHS to obtain 50 µL of a 60 µM dye solution (2.4x protein concentration).
4. Mix $BRD2^{BD2}$ and dye in a 1:1 volume ratio (100 µL final volume, 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 100 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. Centrifuge the eluate 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.
12. Prepare 8 µL aliquots of the labeled BRD2BD2 (~2.5 µM) and immediately store at -80°C.

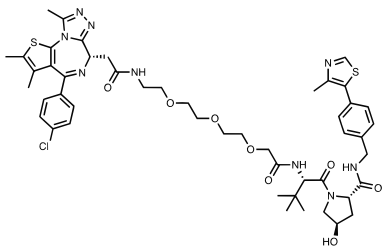
A10. Labeling Efficiency

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

Absorbance A_{280}	0.047	Protein concentration	1.44 µM
Absorbance A_{650}	0.461	Degree-of-labeling (DOL)	1.44

B1. Ligand/Non-Fluorescent Binding Partner

MZ1



Von Hippel-Lindau E3 Ligase (VHL)
uniprot.org/uniprot/P40337

Elongin B (EloB)
uniprot.org/uniprot/Q15370

Elongin C (EloC)
uniprot.org/uniprot/Q15369

B2. Molecule Class/Organism

PROTAC

E3 protein ligase complex
Homo sapiens (Human)

B3. Sequence/Formula

$C_{49}H_{60}ClN_9O_8S_2$

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MPRRAENWDE AEVGAEEAGV EEYGPEEDGG EESGAEESGP EESGPEELGA
EEEMEAGRPR PVLRSVNSRE PSQVIFCNRS PRVVLPVWLN FDGEPQPYPT
LPPGTGRRIH SYRGHLWLFR DAGTHDGLLV NQTELFVPSL NVDGQPIFAN
ITLPVYTLKE RCLQVVRSLV KPENYRRLDI VRSLYEDLED HPNVQKDLER
LTQERIAHQR MGD
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MDVFLMIRRH KTTIFTDAKE SSTVFELKRI VEGILKRPPD EQRLYKDDQL
LDDGKTLGEC GFTSQTARPQ APATVGLAFR ADDTFEALCI EPFSSPPELP
DVMK
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MYVKLISSDG HEFIVKREHA LTSGTIKAML SGPGQFAENE TNEVNFREIP
SHVLSKVCMY FTYKVRYTNS STEIPEFPIA PEIALELLMA ANFLDC
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B4. Purification Strategy/Source

opnme.com
MZ1

Crelux GmbH
Construct ID: ESA2, Lot-ID: PH14288-1

B5. Stock Concentration/Stock Buffer

10 mg/mL | 10 mM
DMSO

10.3 mg/mL | 206 µM
20 mM Bis-Tris/HCl, 150 mM NaCl, 1 mM DTT, pH 7.0

B6. Molecular Weight/Extinction Coefficient

1002.64 Da	24 kDa (VHL), 13.1 kDa (EloB), 12.8 kDa (EloC) 25,900 $M^{-1}cm^{-1}$ (ε_{280})

B7. Serial Dilution Preparation

1. Dissolve 4.9 mg of MZ1 in 489 µL of DMSO to obtain a 10 mM stock solution.
2. Mix 2 µL of 10 mM MZ1 with 98 µL of DMSO to obtain 200 µL of 200 µM MZ1.
3. Mix 4 µL of 200 µM MZ1 with 196 µL of dilution buffer to obtain 200 µL of 4 µM MZ1.
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 $BRD2^{BD2}$ (~2.5 µM) with 496 µL of dilution buffer to obtain 500 µL of ~20 nM $BRD2^{BD2}$.
6. Prepare a 4 µM VCB solution by mixing 2 µL of 206 µM VCB with 101 µL of dilution buffer.

BRD^{BD2} – MZ1 (binary complex)

7. Mix 100 µL of 20 nM $BRD2^{BD2}$ with 100 µL of dilution buffer to obtain 200 µL of 10 nM $BRD2^{BD2}$.
8. Take a fresh 0.5 mL tube and mix 160 µL of 10 nM $BRD2^{BD2}$ with 160 µL of dilution buffer containing 2% DMSO to obtain 320 µL of a 5 nM $BRD2^{BD2}$ solution.
9. Prepare a PCR-rack with 16 PCR tubes. Transfer 20 µL of the 5 nM $BRD2^{BD2}$ solution into tubes **2** to **16**. Then, mix 20 µL of 4 µM MZ1 with 20 µL of 10 nM $BRD2^{BD2}$ 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.

$BRD2^{BD2}$ & high concentration of VCB – MZ1 (ternary complex, saturation)

12. Mix 100 µL of 20 nM $BRD2^{BD2}$ with 50 µL of 4 µM VCB and 50 µL of dilution buffer to obtain 200 µL of 10 nM $BRD2^{BD2}$, 1 µM VCB.
13. Take a fresh 0.5 mL tube and mix 160 µL of 10 nM $BRD2^{BD2}$, 1 µM VCB with 160 µL of dilution buffer containing 2% DMSO to obtain 320 µL of a 5 nM $BRD2^{BD2}$, 500 nM VCB solution.
14. Prepare a PCR-rack with 20 PCR tubes. Transfer 20 µL of the 5 nM $BRD2^{BD2}$, 500 nM VCB solution into tubes **2** to **16**. Then, mix 20 µL of 4 µM MZ1 with 20 µL of 10 nM $BRD2^{BD2}$, 1 µM VCB 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.

$BRD2^{BD2}$ & low concentration of VCB – MZ1 (ternary complex, Hook effect)

17. Mix 100 µL of 20 nM $BRD2^{BD2}$ with 2.5 µL of 4 µM VCB and 97.5 µL of dilution buffer to obtain 200 µL of 10 nM $BRD2^{BD2}$, 50 nM VCB.
18. Take a fresh 0.5 mL tube and mix 160 µL of 10 nM $BRD2^{BD2}$, 50 nM VCB with 160 µL of dilution buffer containing 2% DMSO to obtain 320 µL of a 5 nM $BRD2^{BD2}$, 25 nM VCB solution.
19. Prepare a PCR-rack with 20 PCR tubes. Transfer 20 µL of the 5 nM $BRD2^{BD2}$, 25 nM VCB solution into tubes **2** to **16**. Then, mix 20 µL of 4 µM MZ1 with 20 µL of 10 nM $BRD2^{BD2}$, 50 nM VCB in tube **1**.
20. Prepare a 1:1 serial dilution by transferring 20 µL from tube to tube. Mix carefully by pipetting up and down.
21. Incubate for 5 minutes at room temperature in the dark before loading capillaries.

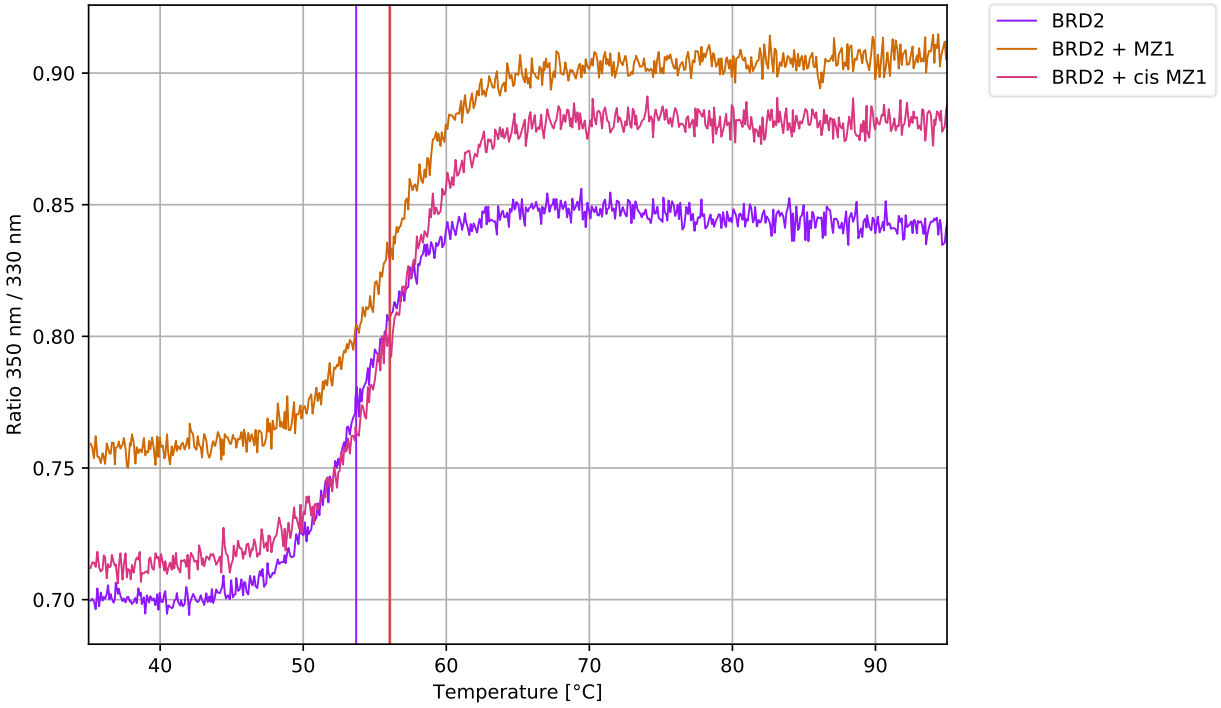
$BRD2^{BD2}$ & low concentration of VCB – cis MZ1 (Control)

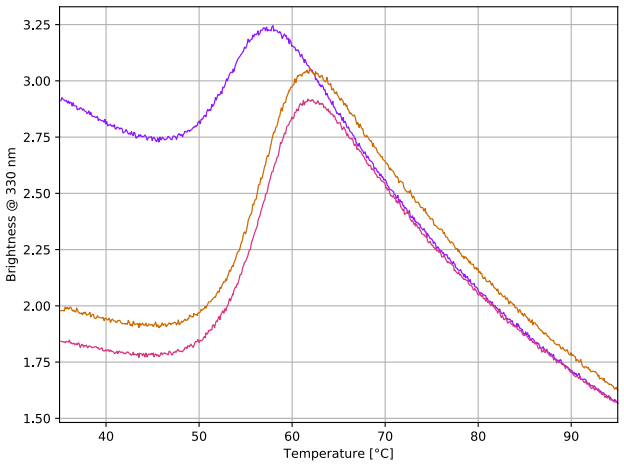
22. Mix 100 µL of 20 nM $BRD2^{BD2}$ with 2.5 µL of 4 µM VCB and 97.5 µL of dilution buffer to obtain 200 µL of 10 nM $BRD2^{BD2}$, 50 nM VCB.
23. Take a fresh 0.5 mL tube and mix 160 µL of 10 nM $BRD2^{BD2}$, 50 nM VCB with 160 µL of dilution buffer containing 2% DMSO to obtain 320 µL of a 5 nM $BRD2^{BD2}$, 25 nM VCB solution.
24. Prepare a PCR-rack with 20 PCR tubes. Transfer 20 µL of the 5 nM $BRD2^{BD2}$, 25 nM VCB solution into tubes **2** to **16**. Then, mix 20 µL of 4 µM cis MZ1 with 20 µL of 10 nM $BRD2^{BD2}$, 50 nM VCB in tube **1**.
25. Prepare a 1:1 serial dilution by transferring 20 µL from tube to tube. Mix carefully by pipetting up and down.
26. Incubate for 5 minutes at room temperature in the dark before loading capillaries.

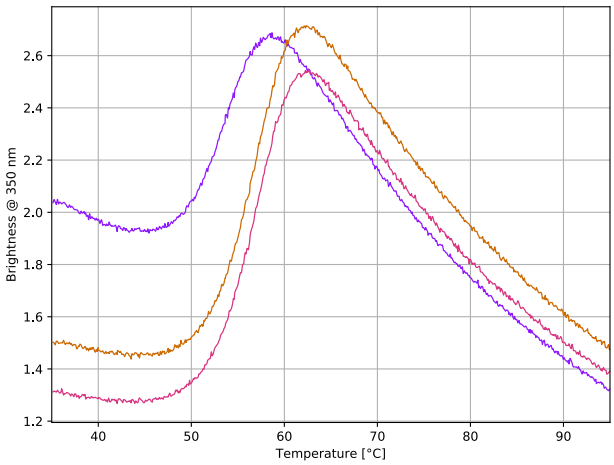
C. Tycho

Validation of structural integrity and functionality of $BRD2^{BD2}$ using Tycho NT.6:
nanotempertech.com/tycho

BRD2	5 µL of 4 µM $BRD2^{BD2}$ + 5 µL of dilution buffer containing 2% DMSO	T_i = 53.7°C
BRD2 + MZ1	5 µL of 4 µM $BRD2^{BD2}$ + 5 µL of 4 µM MZ1	T_i = 56.1°C
BRD2 + cis MZ1	5 µL of 4 µM $BRD2^{BD2}$ + 5 µL of 4 µM cis MZ1	T_i = 56.0°C

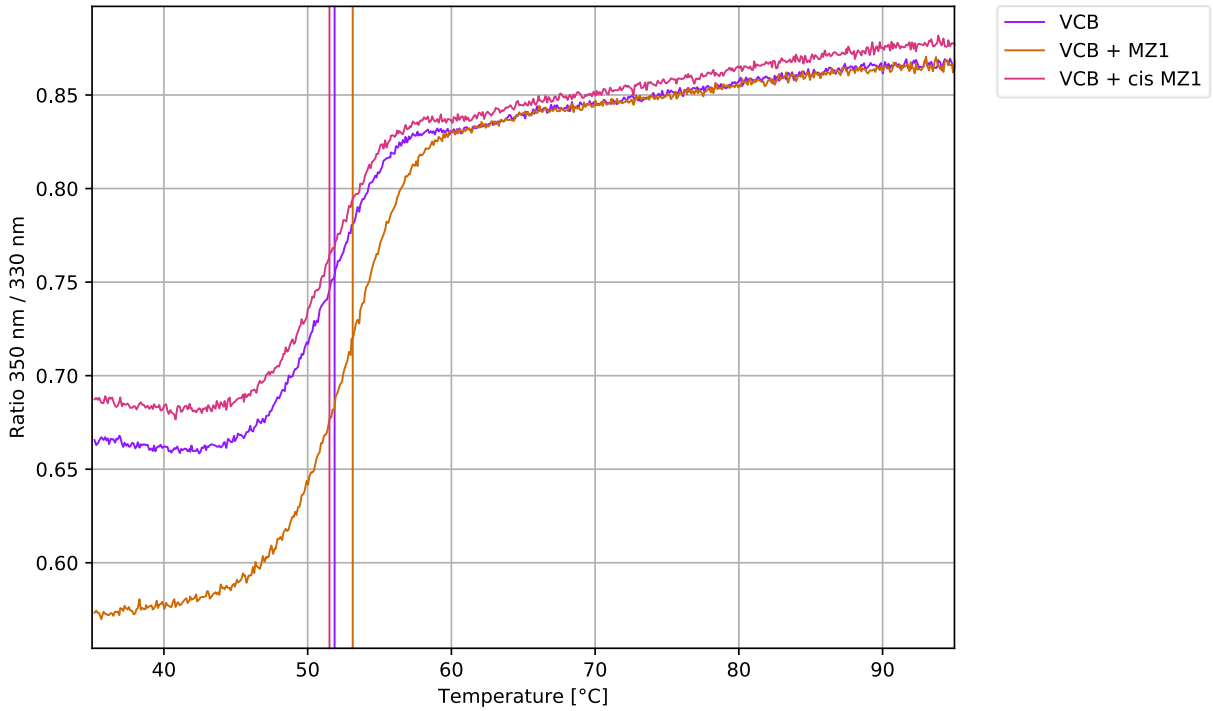


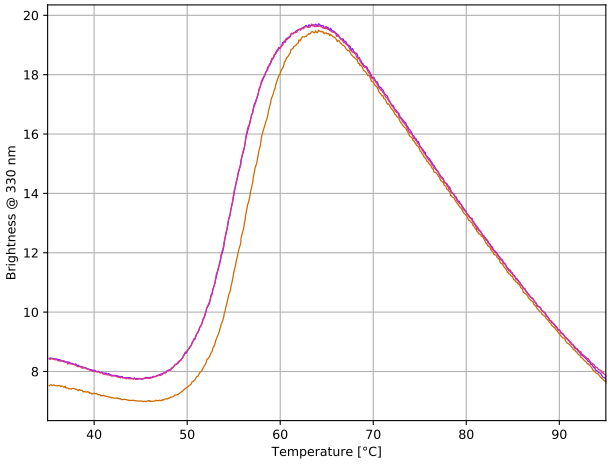


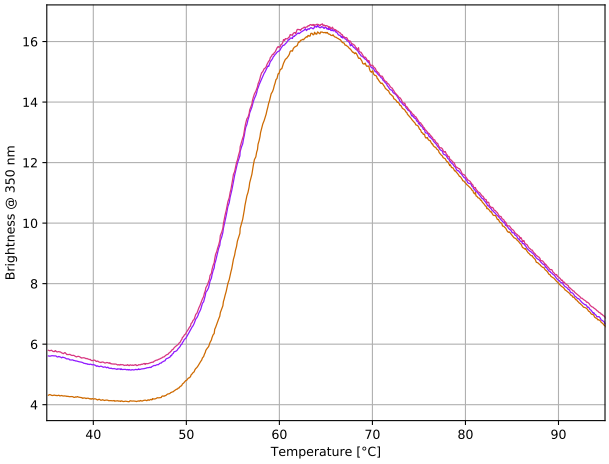


Validation of structural integrity and functionality of VCB using Tycho NT.6:
nanotempertech.com/tycho

VCB	5 µL of 4 µM VCB + 5 µL of dilution buffer containing 2% DMSO	T_i = 51.9°C
VCB + MZ1	5 µL of 4 µM VCB + 5 µL of 4 µM MZ1	T_i = 53.1°C
VCB + cis MZ1	5 µL of 4 µM VCB + 5 µL of 4 µM cis MZ1	T_i = 51.5°C







D1. Monolith System/Capillaries

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

D2. Monolith Software

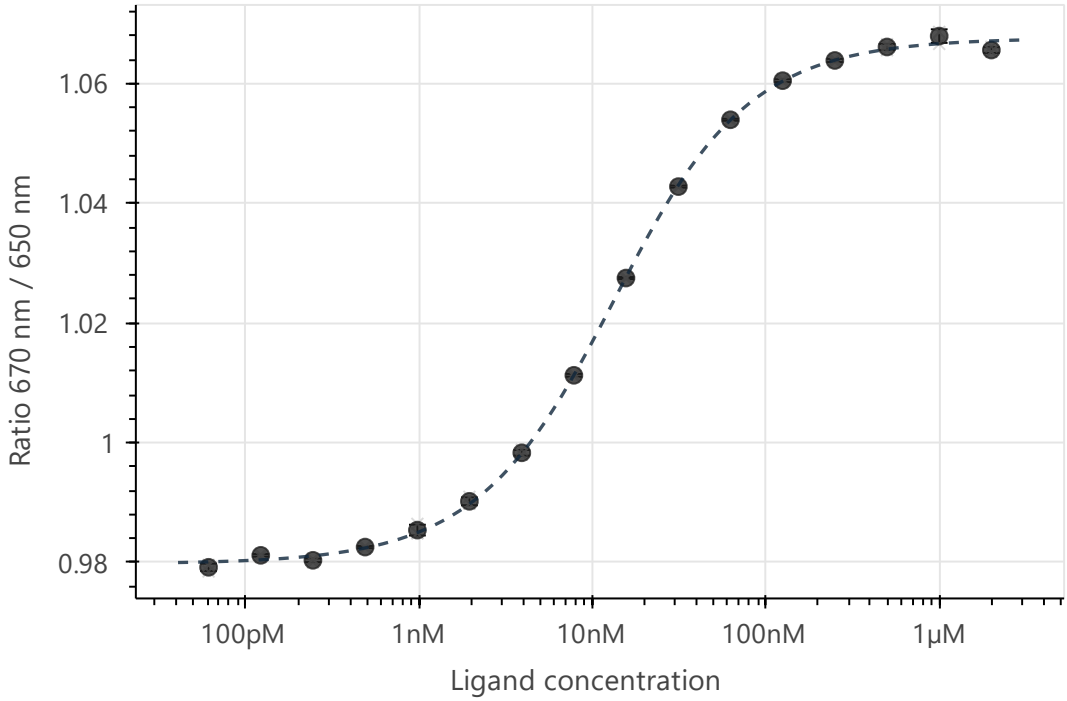
MO.Control v2.4.2 (NanoTemper Technologies GmbH)
nanotempertech.com/monolith-mo-control-software

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

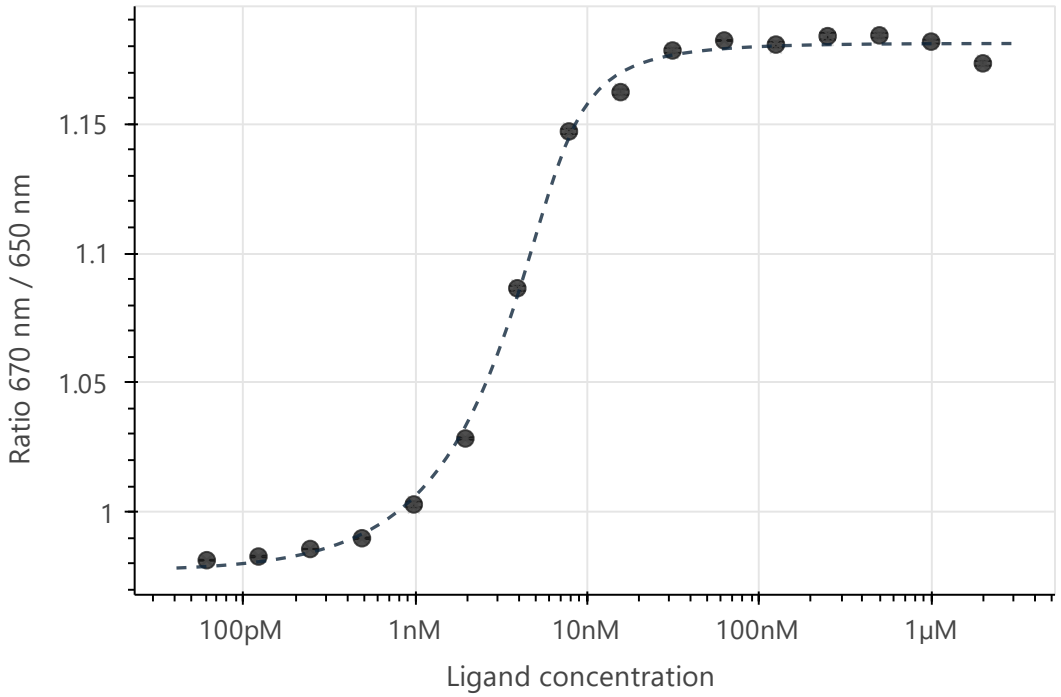
50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM GSH, 0.01 % Pluronic® F-127, 1% DMSO
5 nM $BRD2^{BD2}$ | 2 µM – 61 pM MZ1 | 20°C | 100% Excitation Power

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

$BRD2^{BD2}$ – MZ1 (binary complex) | K_d = 10.7 ± 0.2 nM (S/N = 132.3)

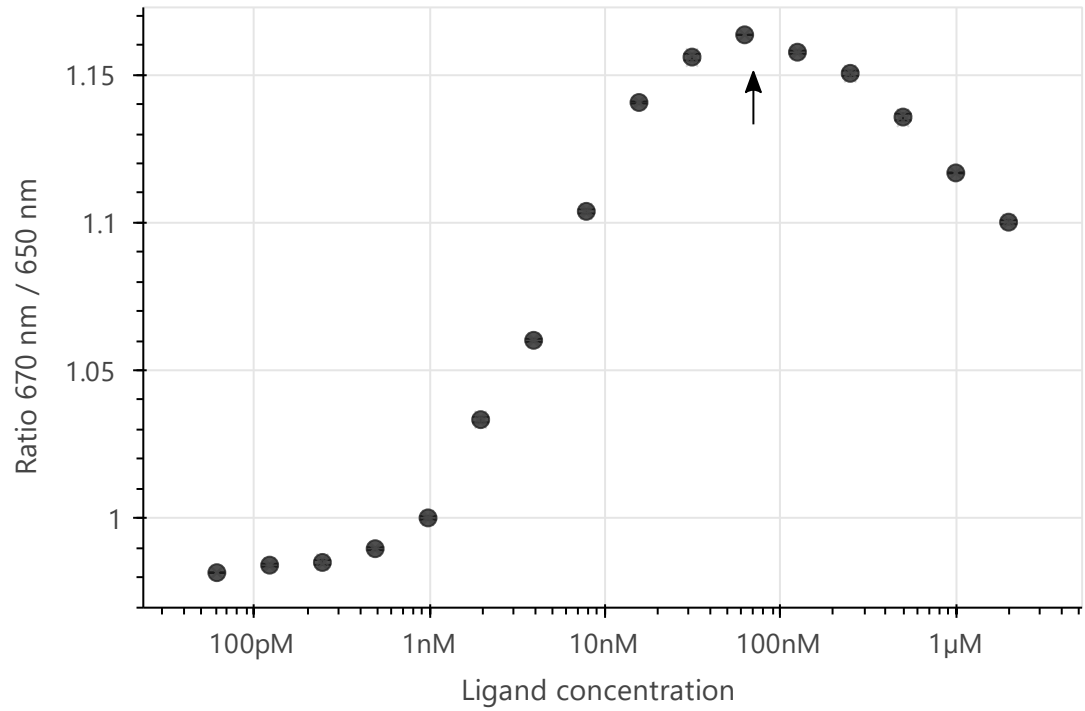


BRD2^{BD2} & VCB (500 nM) – MZ1 (ternary complex, saturation) | K_d = 566 ± 94 pM (S/N = 54.4)

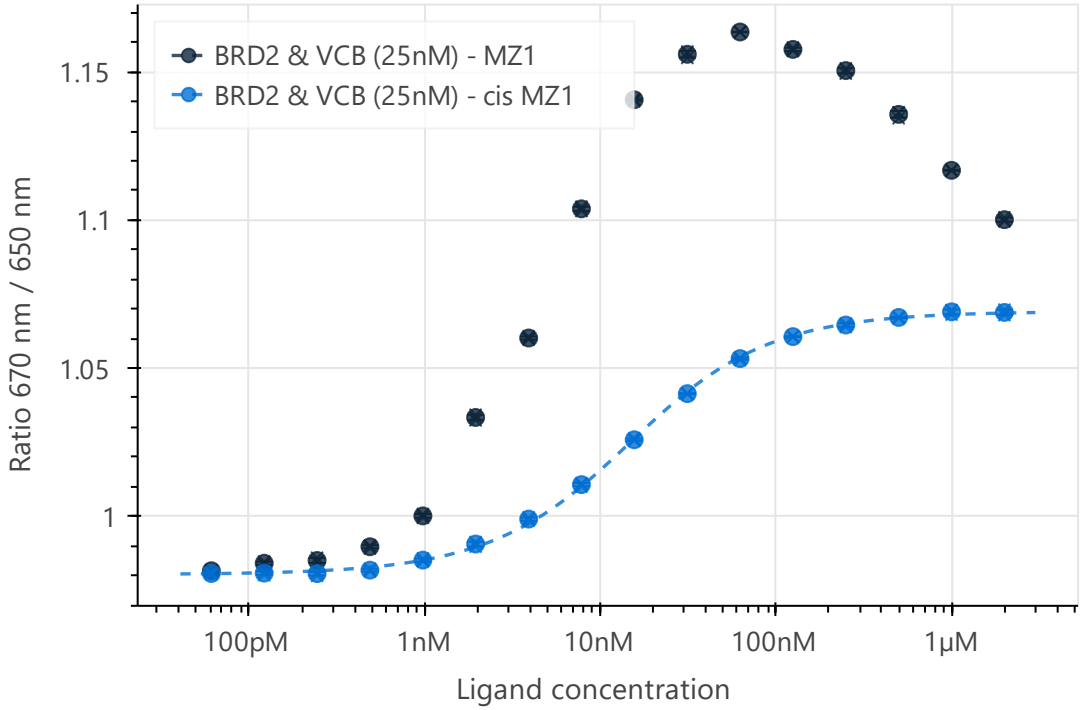


Cooperativity factor: $\alpha = \frac{10.7\ \text{nM}}{0.566\ \text{nM}} \approx 18.9$

BRD2^{BD2} & VCB (25 nM) – MZ1 (ternary complex, Hook effect)

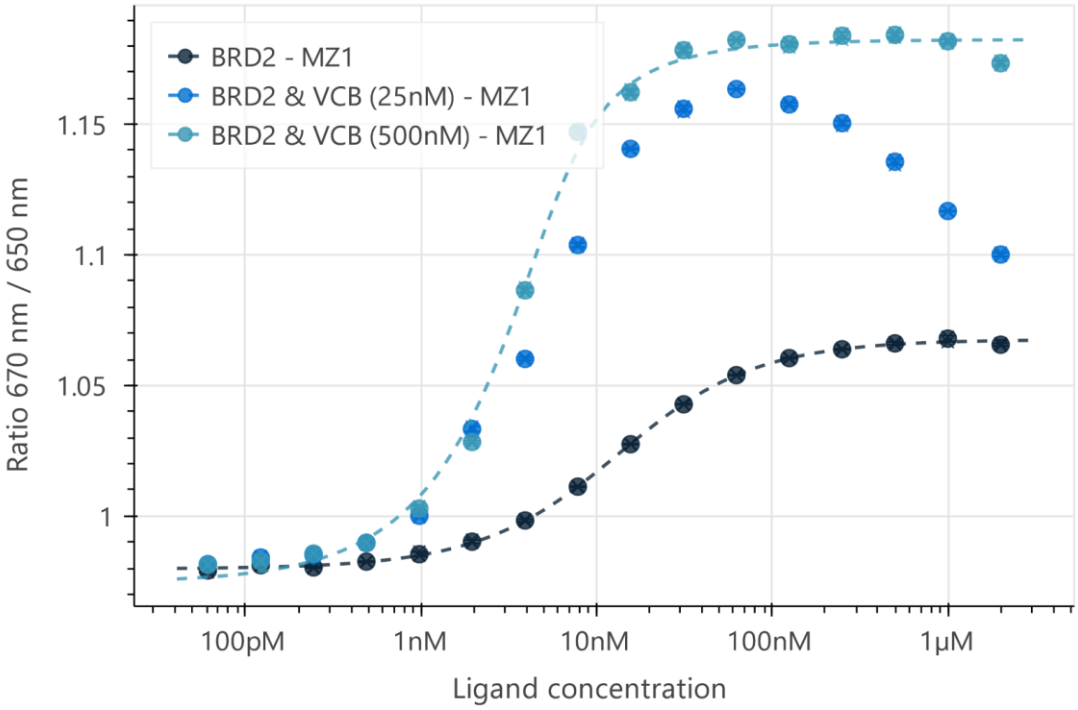


BRD2^{BD2} & VCB (25 nM) – cis MZ1 (control) | K_d = 12.2 ± 0.4 nM (S/N = 124)



No Hook effect and ternary complex formation

Overlay plot:



D5. Reference Results/Supporting Results

PROTAC		+ target	Monolith X		ITC	
			K_d (nM)	α[1]	K_d (nM)	α
MZ1	binary	-	10.7	-	60	-
MZ1	ternary	VCB	0.566	18.9	-	-

Isothermal Titration Calorimetry (ITC)
Gadd et al., Nat Chem Biol 13, 514–521 (2017)

E. Contributors

Andreas Langer[2]

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