NANOTEMPER

Monolith X Protocol MOX-P-102

VCB (VHL) E3 Ligase – MZ1 (PROTAC) – BRD2[BD2]

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

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

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

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

A2. Molecule Class/Organism

E3 protein ligase complex
Homo sapiens (Human)

A3. Sequence/Formula

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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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A4. Purification Strategy/Source

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

A5. Stock Concentration/Stock Buffer

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

A6. Molecular Weight/Extinction Coefficient

24 kDa (VHL), 13.1 kDa (EloB), 12.8 kDa (EloC)
25,900 $M^{-1}cm^{-1}$ (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 – MALEIMIDE 2nd Generation[1] (MO-L014, NanoTemper Technologies GmbH)
1* Labeling Buffer Maleimide | 1* Dye RED-MALEIMIDE 2nd Generation (10 µg) | 1* A-Column | 1* B-Column

A9. Labeling Procedure

1. Prepare 50 µL of 25 µM VCB (VHL/Elongin B/Elongin C) by mixing 6.1 µL of 206 µM VCB with 43.9 µL of Labeling Buffer Maleimide.
2. Use the A-Column to perform a buffer exchange into Labeling Buffer Maleimide.
    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 Labeling Buffer Maleimide and centrifuge at **1500 × g** for **1 min** (3x).
    e. Place 50 µL of the 25 µM VCB 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 50 µL of ~20 µM VCB (~80% recovery).
3. Add 22 µL of DMSO to Dye RED-MALEIMIDE 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 5 µL of the 600 µM dye solution with 45 µL of Labeling Buffer Maleimide to obtain 50 µL of a 60 µM dye solution (3x protein concentration).
5. Mix VCB and dye in a 1:1 volume ratio (100 µL final volume, 5% final DMSO concentration).
6. Incubate for 45 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 100 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. Centrifuge the eluate at 15,000 rpm and 4°C for 20 minutes to remove aggregates. Then, carefully transfer the supernatant into a fresh tube and mix well by pipetting.
13. Prepare 8 µL aliquots of the labeled VCB (~2.5 µM) and immediately store at -80°C.

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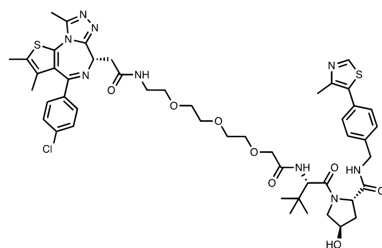
[1] While Cys162 of VHL is buried at the interface between VHL and EloC, Cys77 is solvent exposed and adjacent to the native substrate binding site. Cys89 of EloB is also solvent exposed.

A10. Labeling Efficiency

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

Absorbance A_{280}	0.097	Protein concentration	2.51 µM
Absorbance A_{650}	0.797	Degree-of-labeling (DOL)	1.63

B1. Ligand/Non-Fluorescent Binding Partner

MZ1 	BRD2[BD2] uniprot.org/uniprot/P25440

B2. Molecule Class/Organism

PROTAC	Bromo-domain containing protein

B3. Sequence/Formula

$C_{49}H_{60}ClN_9O_8S_2$	MHHHHHHSGV DLGTENLYFQ SMGKLSEQLK HCNGILKELL SKKHAAYAWP FYKPVDASAL GLHDYHDIIK HPMDLSTVKR KMENRDYRDA QEFAADVRLM FSNCYKYNPP DHDVVAMARK LQDVFEFRYA KMPD

B4. Purification Strategy/Source

opnme.com MZ1	Crelux GmbH Construct ID: CRN1, Lot-ID: PC16670

B5. Stock Concentration/Stock Buffer

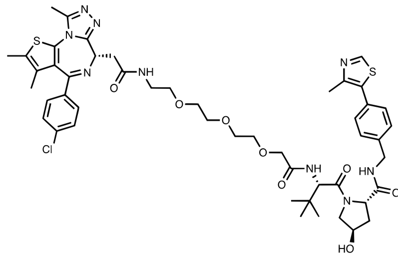
10 mg/mL | 10 mM DMSO	5.72 mg/mL | 362 µM 25 mM HEPES pH 7.5, 100 mM NaCl, 5% glycerol, 1 mM DTT

B6. Molecular Weight/Extinction Coefficient

1002.64 Da	15.8 kDa 17,420 $M^{-1}cm^{-1}$ (ε_{280})

B1. Ligand/Non-Fluorescent Binding Partner

MZ1



BRD2^{BD2}

uniprot.org/uniprot/P25440

B2. Molecule Class/Organism

PROTAC

Bromo-domain containing protein

B3. Sequence/Formula

$C_{49}H_{60}ClN_9O_8S_2$

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

B4. Purification Strategy/Source

opnme.com
MZ1

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

B5. Stock Concentration/Stock Buffer

10 mg/mL | 10 mM
DMSO

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

B6. Molecular Weight/Extinction Coefficient

1002.64 Da

15.8 kDa
17,420 $M^{-1}cm^{-1}$ (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 100 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 VCB (~2.5 µM) with 496 µL of dilution buffer to obtain 500 µL of ~20 nM VCB.
6. Prepare a 4 µM $BRD2^{BD2}$ solution by mixing 2 µL of 362 µM BRD2 with 179 µL of dilution buffer.

VCB – MZ1 (binary complex)

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

VCB – cis MZ1 (Control)

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

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

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

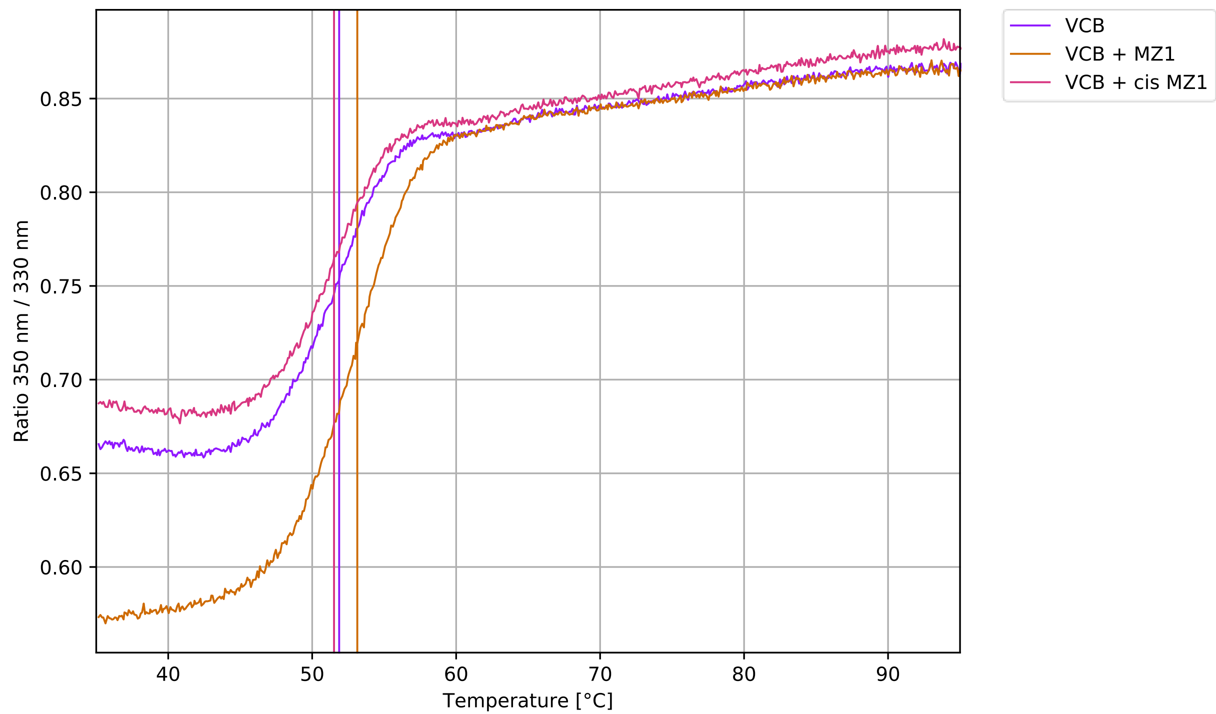
VCB & low concentration of BRD2^{BD2} – MZ1 (ternary complex, Hook effect)

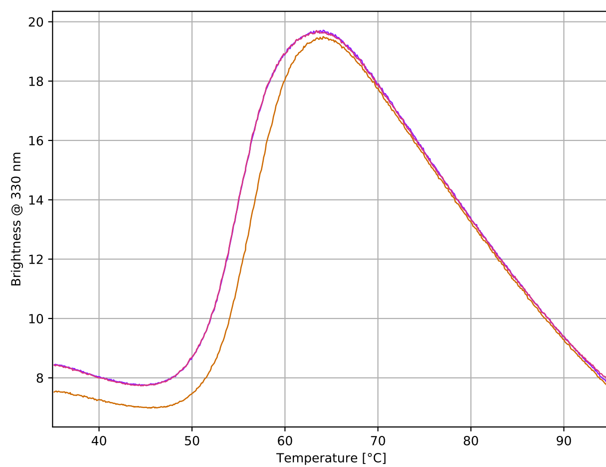
22. Mix 100 µL of 20 nM VCB with 2.5 µL of 4 µM BRD2^{BD2} and 97.5 µL of dilution buffer to obtain 200 µL of 10 nM VCB, 50 nM BRD2^{BD2}.
23. Take a fresh 0.5 mL tube and mix 160 µL of 10 nM VCB, 50 nM BRD2^{BD2} with 160 µL of dilution buffer containing 2% DMSO to obtain 320 µL of a 5 nM VCB, 25 nM BRD2^{BD2} solution.
24. Prepare a PCR-rack with 20 PCR tubes. Transfer 20 µL of the 5 nM VCB, 25 nM BRD2^{BD2} solution into tubes **2** to **16**. Then, mix 20 µL of 4 µM MZ1 with 20 µL of 10 nM VCB, 50 nM BRD2^{BD2} 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.

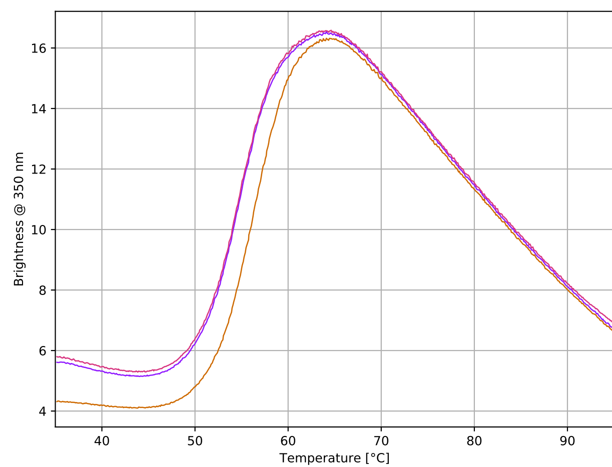
C. Tycho

Validation of structural integrity and functionality of 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

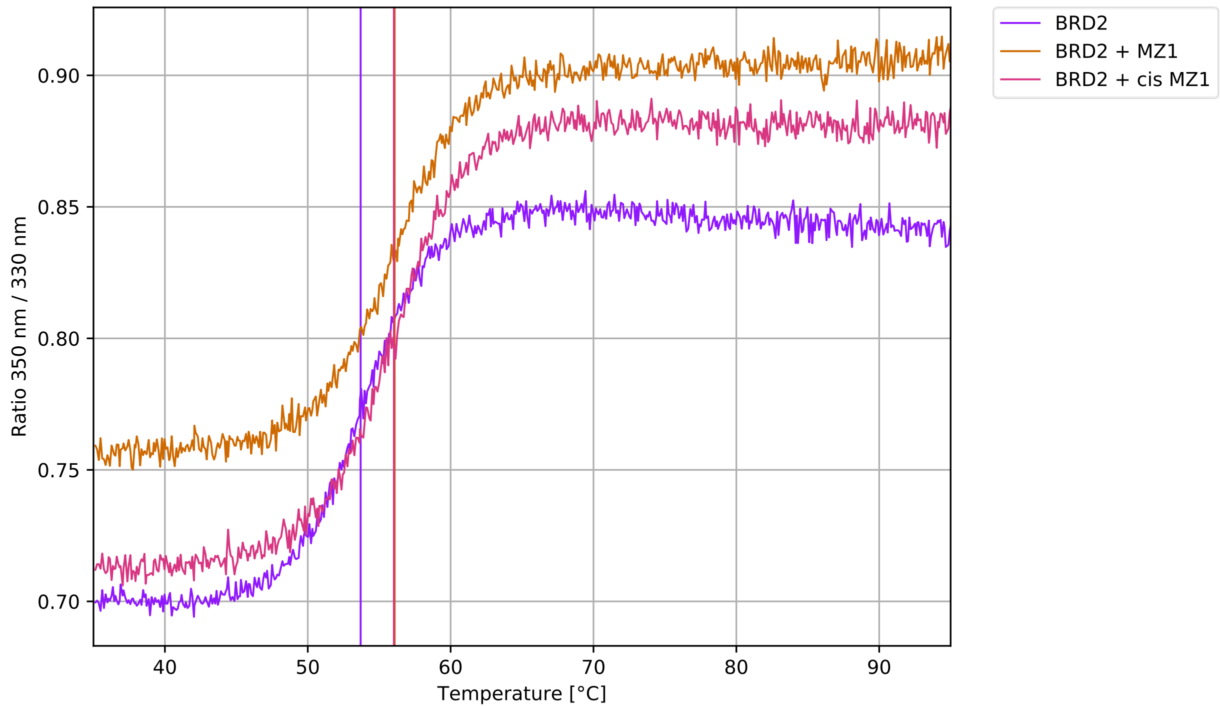


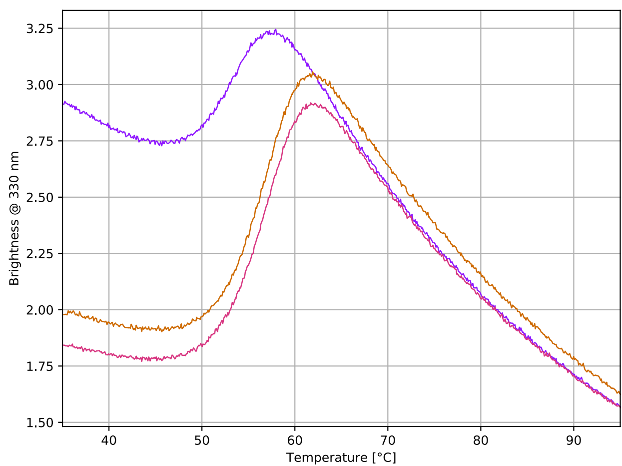


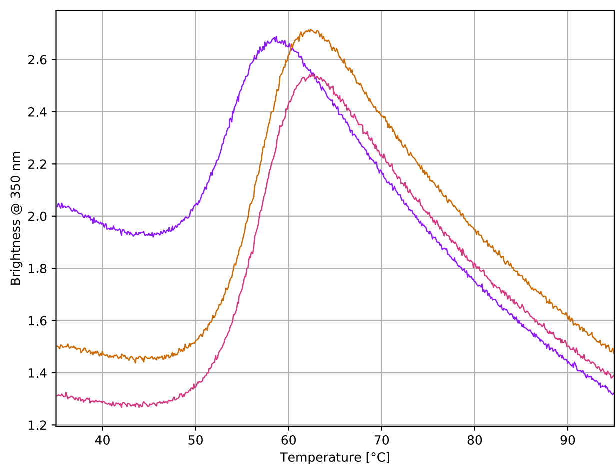


Validation of structural integrity and functionality of BRD2^{BD2} using Tycho NT.6:
nanotempertech.com/tycho

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







D1. Monolith System/Capillaries

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

D2. Monolith Software

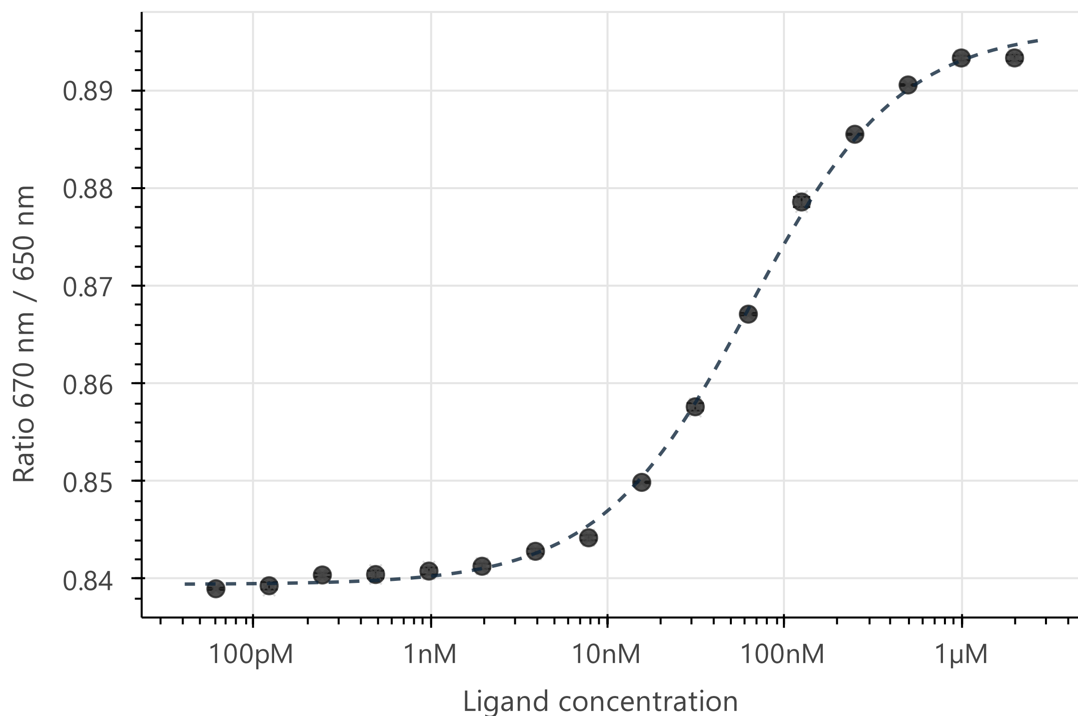
MO.Control v2.4.2 (NanoTemper Technologies GmbH)
nanotempertech.com/monolith-mo-control-software

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

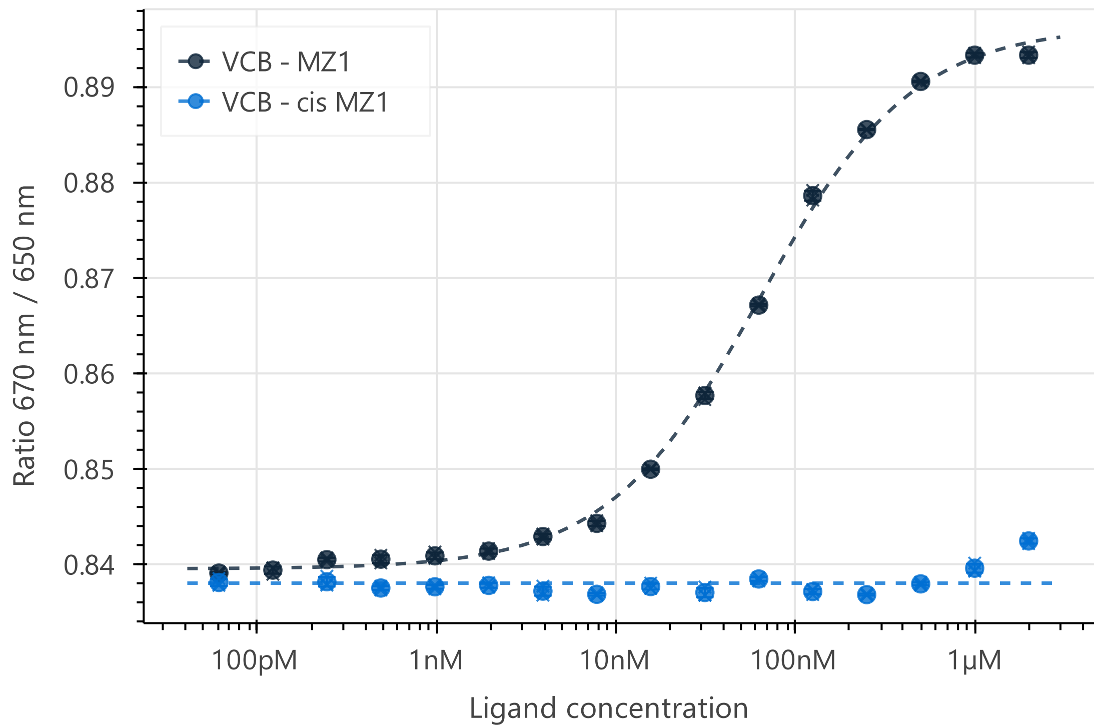
50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM GSH, 0.01 % Pluronic® F-127, 1% DMSO
5 nM VCB | 2 µM – 61 pM MZ1 | 20°C | 100% Excitation Power

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

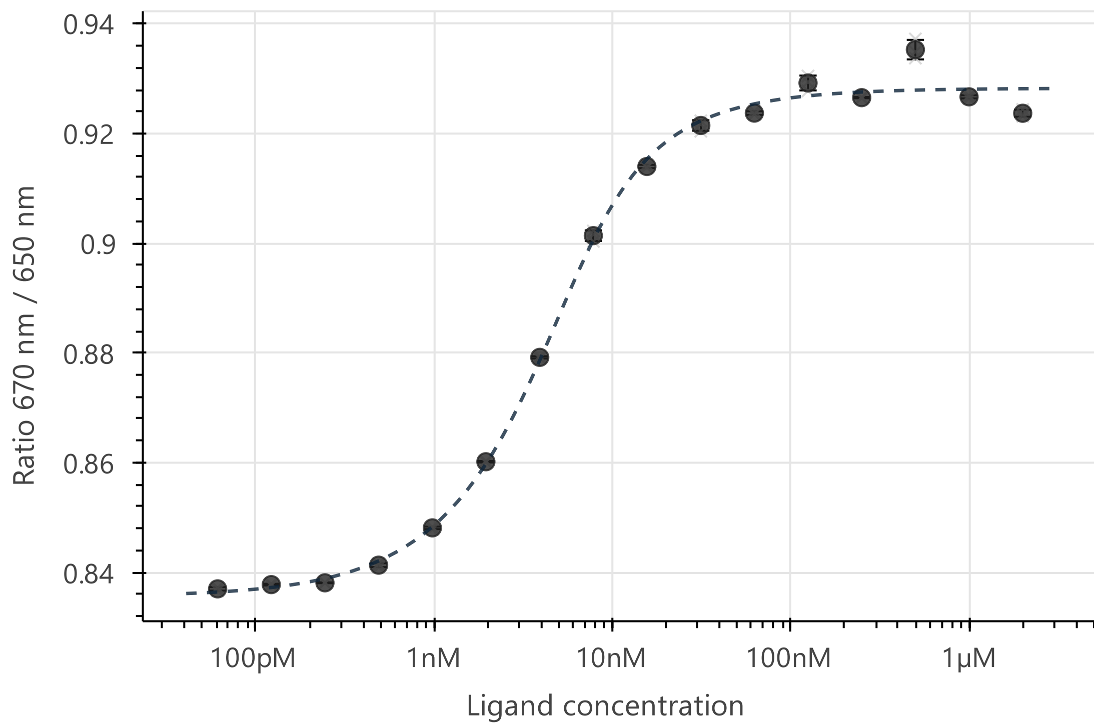
VCB – MZ1 (binary complex) | K_d = 61.2 ± 2.8 nM (S/N = 76.1)



VCB – cis MZ1 (control) | K_d > 10 µM

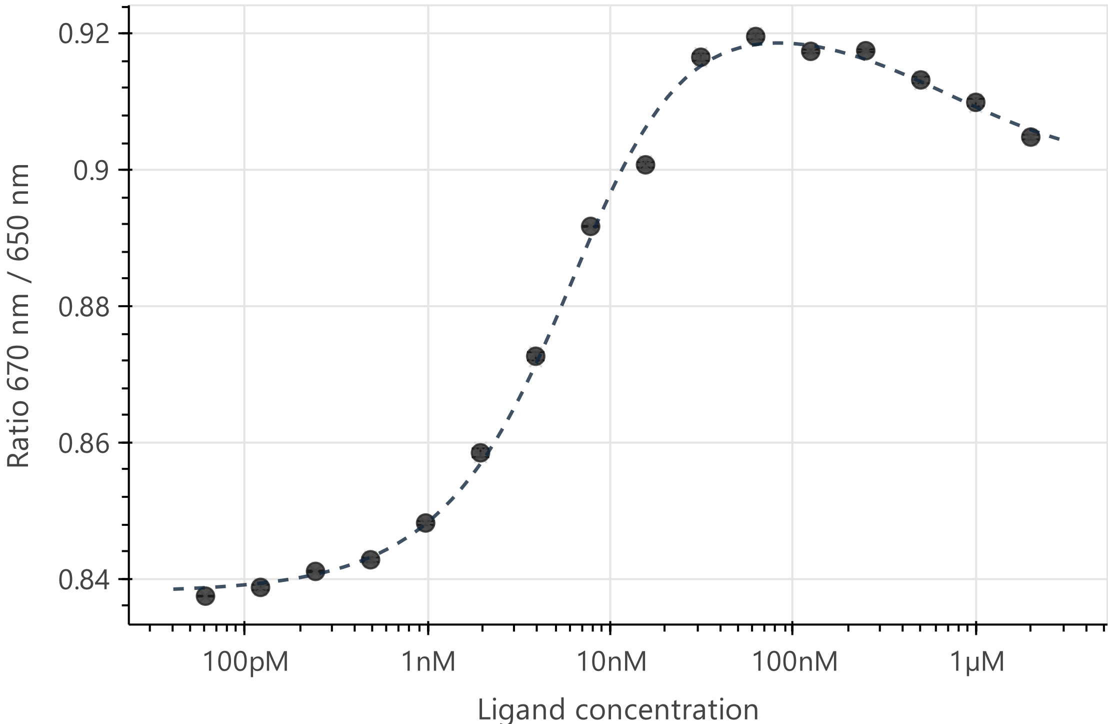


VCB & BRD2[BD2] (500 nM) – MZ1 (ternary complex, saturation) | K_d = 1.83 ± 0.25 nM (S/N = 37.5)

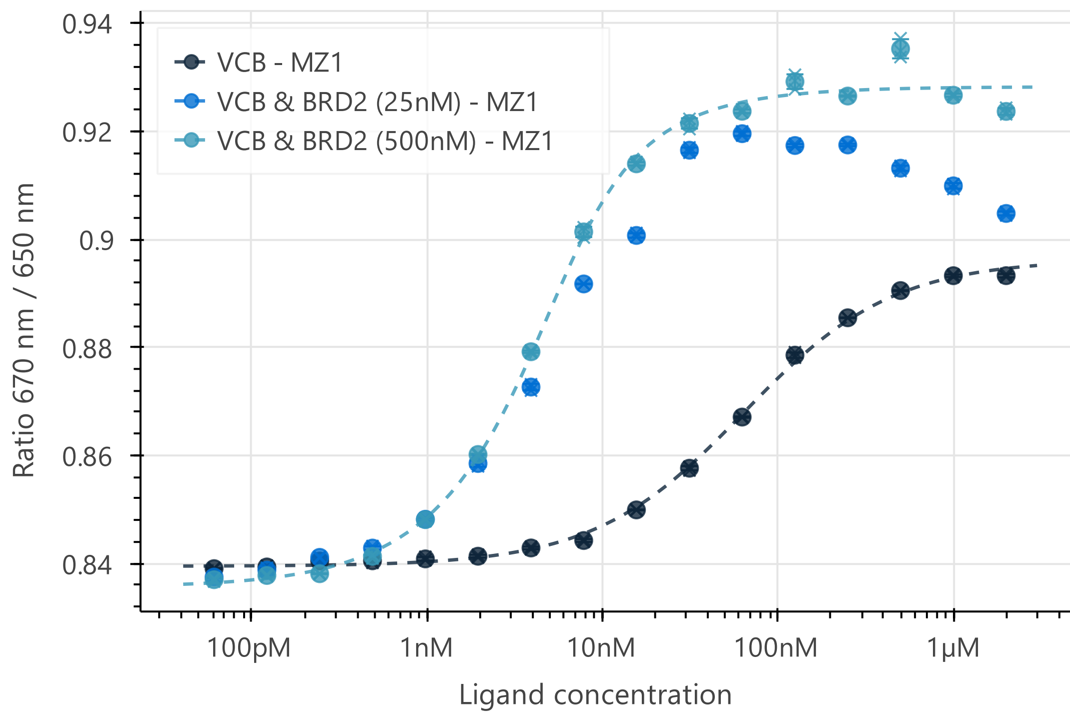


Cooperativity factor: $\alpha = \frac{61.2 \text{ nM}}{1.83 \text{ nM}} \approx 33.4$

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



Overlay



D5. Reference Results/Supporting Results

PROTAC		+ target	Monolith X		SPR		ITC	
			K_d (nM)	α[2]	K_d (nM)	α	K_d (nM)	α
MZ1	binary	-	61.2	-	29	-	66	-
MZ1	ternary	$BRD2^{BD2}$	1.83	33.4	0.9	32	28	2.3

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

Surface Plasmon Resonance (SPR)
Roy et al., ACS Chem. Biol. 14, 361–368 (2019)

E. Contributors

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