Monolith X Protocol MOX-P-100

p38-alpha – Inhibitors

Mitogen-activated protein kinase 14 (MAPK14, also called p38-α) is an enzyme that has been implicated in the regulation of many proinflammatory pathways. It is a drug target for many diseases such as rheumatoid arthritis, endotoxic shock and osteoporosis. SB 202190, SB 203580, SB 239063, PD 169316 and RWJ 67657 are specific and potent inhibitors of kinases that bind p38-α with low nanomolar affinity. BIRB 796 belongs to a novel class of allosteric inhibitors and binds p38-α with picomolar affinity.

protein – small molecule | kinase | inhibitor

A1. Target/Fluorescent Molecule

Mitogen-activated protein kinase 14 (p38-α)
uniprot.org/uniprot/Q16539

A2. Molecule Class/Organism

Mitogen-activated protein kinase (MAP kinase)
Homo sapiens (Human)

A3. Sequence/Formula

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MSYYHHHHHH DYDIPTTENL YFQGAMWMSQ ERPTFYRQEL NKTIWEVPER YQNLSPVGSG AYGSVCAAFD TKTGLRVAVK
KLSRPFQSII HAKRTYRELR LLKHMKHENV IGLLDVFTPA RSLEEFNDVY LVTHLMGADL NNIVKCQKLT DDHVQFLIYQ
ILRGLKYIHS ADIIHRDLKP SNLAVNEDCE LKILDFGLAR HTDDEMTGYV ATRWYRAPEI MLNWMHYNQT VDIWSVGCIM
AELLTGRTLF PGTDHIDQLK LILRLVGTPG AELLKKISSE SARNYIQSLT QMPKMNFANV FIGANPLAVD LLEKMLVLDS
DKRITAAQAL AHAYFAQYHD PDDEPVADPY DQSFESRDLL IDEWKSLTYD EVISFVPPPL DQEEMES
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A4. Purification Strategy/Source

Expressed in E. coli BL21 (DE3), Lot PC11655-3, Construct CJA3
Crelux GmbH

A5. Stock Concentration/Stock Buffer

2.2 mg/mL | 50 µM
25 mM HEPES, pH 7.4, 50 mM NaCl, 10 mM DTT, 1 mM EDTA

A6. Molecular Weight/Extinction Coefficient

44.7 kDa
50,100 $M^{-1}cm^{-1}$ (ε_{280})

A7. Dilution Buffer

20 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM GSH, 1 mM EDTA, 0.02% Pluronic® F-127

A8. Labeling Strategy

Monolith Protein Labeling Kit RED – MALEIMIDE 2nd Generation (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 p38-α.
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 p38-α 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**.

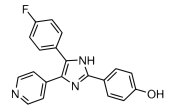
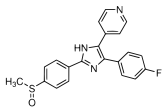
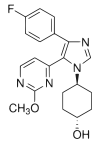
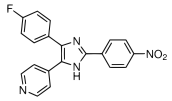
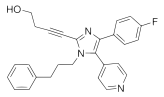
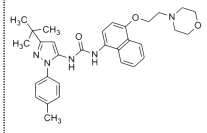
   The collected flow-through should yield around 50 µL of ~20 µM p38-α (~80% recovery).
3. Add 22 µL of DMSO to Dye RED-MALEIMIDE 2nd Generation (10 µg) to obtain a ~600 µM solution. Mix the dye thoroughly by vortexing and make sure that all dye is dissolved.
4. Mix 4 µL of the 600 µM dye solution with 46 µL of Labeling Buffer Maleimide to obtain 50 µL of a 48 µM dye solution (2.4x protein concentration).
5. Mix p38-α and dye in a 1:1 volume ratio (100 µL final volume, 4% 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 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 500 µL of dilution buffer and collect the eluate.
12. Centrifuge the eluate at 15,000 rpm and 4°C for 15 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 p38-α (~2 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}	0.083	Protein concentration	1.50 µM
Absorbance A_{650}	0.202	Degree-of-labeling (DOL)	0.69

B1. Ligand/Non-Fluorescent Binding Partner

SB 202190	SB 203580	SB 239063	PD 169316	RWJ 67657	BIRB 796
					

B2. Molecule Class/Organism

p38 MAPK inhibitors

B3. Sequence/Formula

$C_{20}H_{14}FN_3O$	$C_{21}H_{16}FN_3OS$	$C_{20}H_{21}N_4O_2F$	$C_{20}H_{13}FN_4O_2$	$C_{27}H_{24}FN_3O$	$C_{31}H_{37}N_5O_3$

B4. Purification Strategy/Source

Sigma-Aldrich GmbH

S7067	S8307	S0569	P9248	SML2212	506172

B5. Stock Concentration/Stock Buffer

1 mg/mL in DMSO

3.02 mM	2.65 mM	2.71 mM	2.78 mM	2.35 mM	1.90 mM

B6. Molecular Weight/Extinction Coefficient

331.34 Da	377.43 Da	368.40 Da	360.34 Da	425.50 Da	527.66 Da

B7. Serial Dilution Preparation

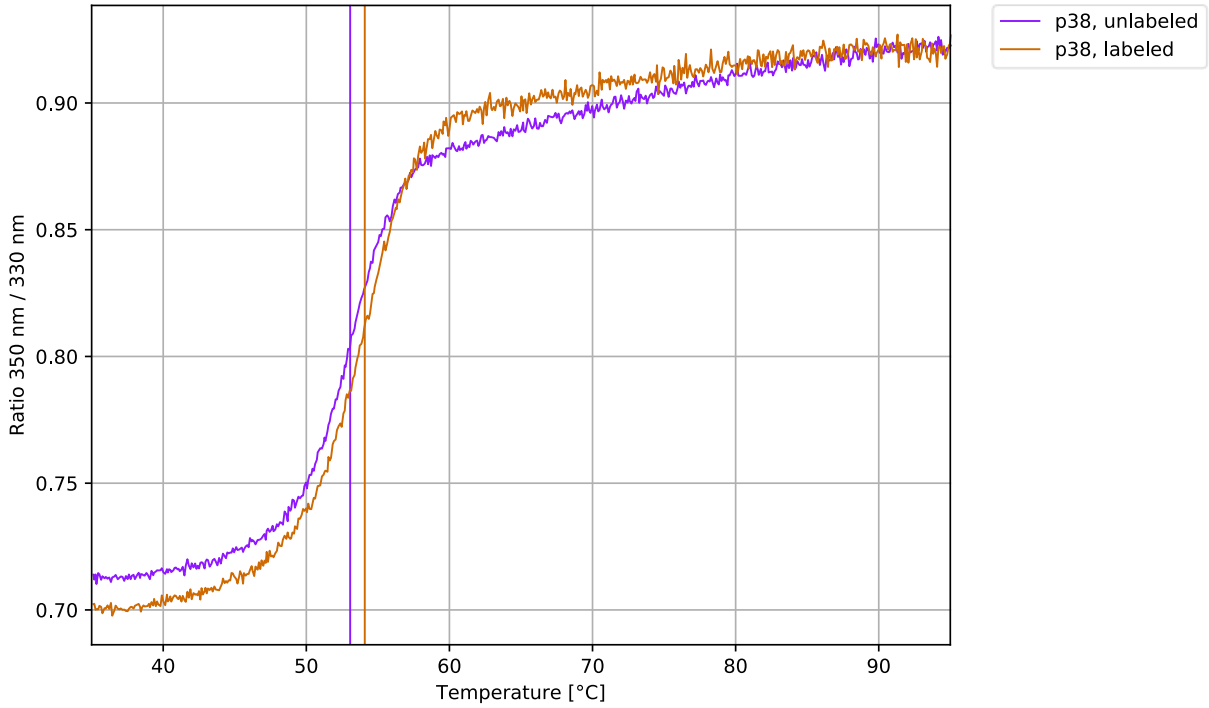
1. Dilute inhibitor stock in DMSO to a 500 µM solution.
2. Mix 2 µL of the 500 µM inhibitor solution with 98 µL of dilution buffer to obtain 100 µL of a 10 µM compound solution containing 2% DMSO.
3. Mix 20 µL of DMSO with 980 µL of dilution buffer to obtain 1 mL of a 2% DMSO solution.
4. Prepare a PCR-rack with 16 PCR tubes. Transfer 20 µL of the 10 µM compound solution into tube **1**. Then, transfer 10 µL of the 2% DMSO solution into tubes **2** to **16**.
5. Prepare a 1:1 serial dilution by transferring 10 µL from tube to tube. Mix carefully by pipetting up and down. Remember to discard 10 µL from tube **16** to get an equal volume of 10 µL for all samples.
6. Mix 2 µL of labeled p38-α (~2 µM) with 198 µL of dilution buffer to obtain 200 µL of ~20 nM p38-α.
7. Add 10 µL of this solution to each tube from **16** to **1** and mix by pipetting.
8. Incubate for 30 minutes[1] at room temperature in the dark before loading capillaries.

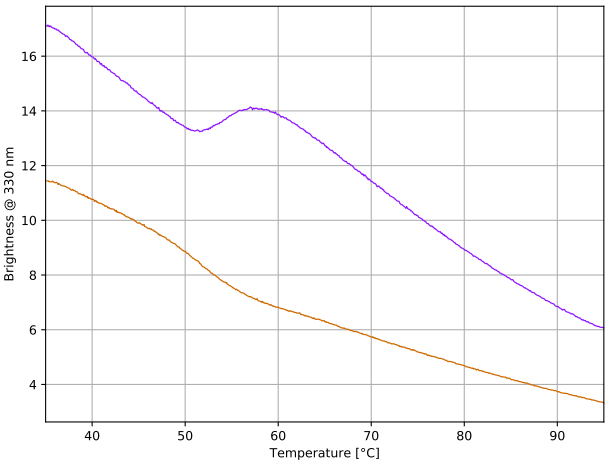
[1] At least 3 hours for BIRB 796.

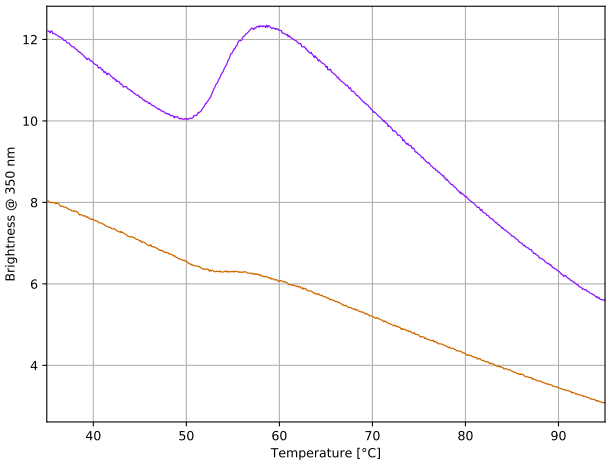
C. Tycho

Validation of structural integrity of labeled p38-α using Tycho NT.6:
nanotempertech.com/tycho

p38-α, unlabeled	5 µL of 2 µM p38-α + 5 µL of dilution buffer with 2% DMSO	T_i = 53.1°C
p38-α, labeled	5 µL of B-Column eluate (~2 µM) + 5 µL of dilution buffer with 2% DMSO	T_i = 54.1°C



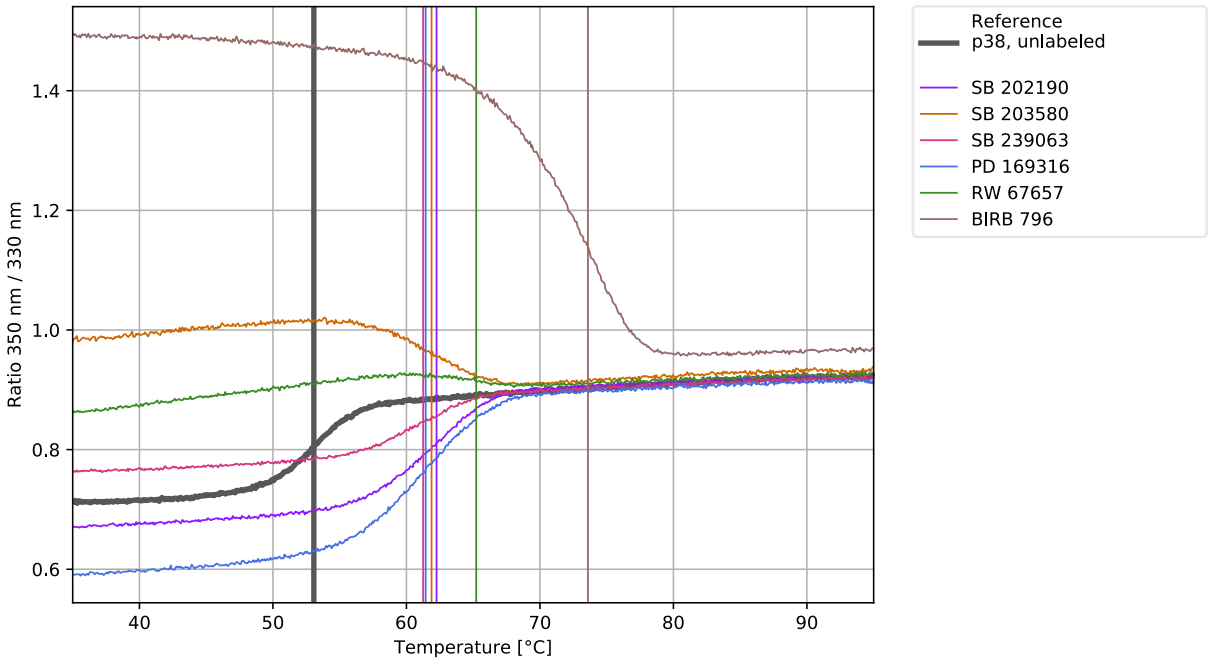


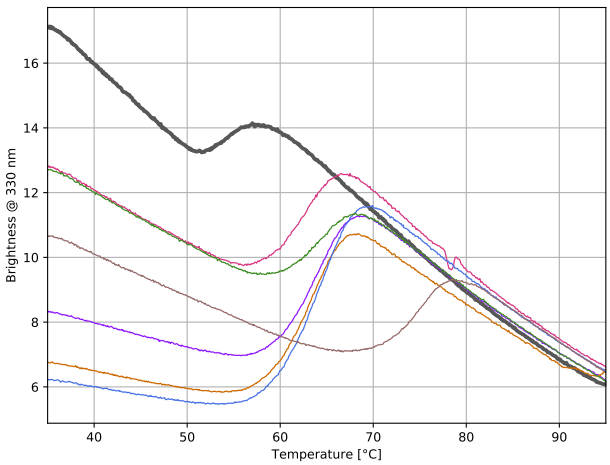


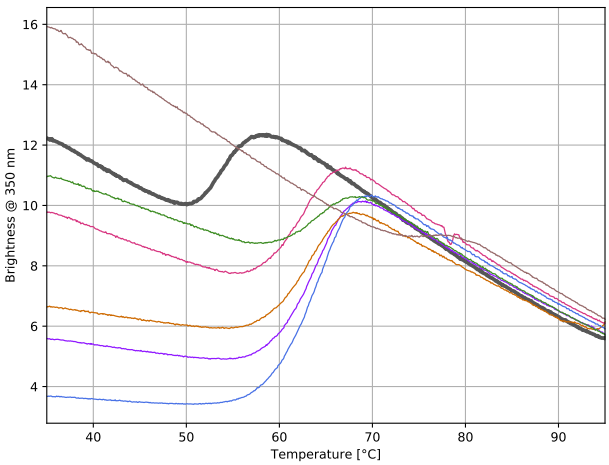
Validation of functionality of p38-α using Tycho NT.6:

nanotempertech.com/tycho

SB 202190	5 µL of 2 µM p38-α + 5 µL of 10 µM SB 202190	T_i = 62.3°C
SB 203580	5 µL of 2 µM p38-α + 5 µL of 10 µM SB 203580	T_i = 61.9°C
SB 239063	5 µL of 2 µM p38-α + 5 µL of 10 µM SB 239163	T_i = 61.3°C
PD 169316	5 µL of 2 µM p38-α + 5 µL of 10 µM PD 169316	T_i = 61.4°C
RWJ 67657	5 µL of 2 µM p38-α + 5 µL of 10 µM RWJ 67657	T_i = 65.2°C
BIRB 796	5 µL of 2 µM p38-α + 5 µL of 10 µM BIRB 796	T_i = 73.6°C







D1. Monolith System/Capillaries

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

D2. Monolith Software

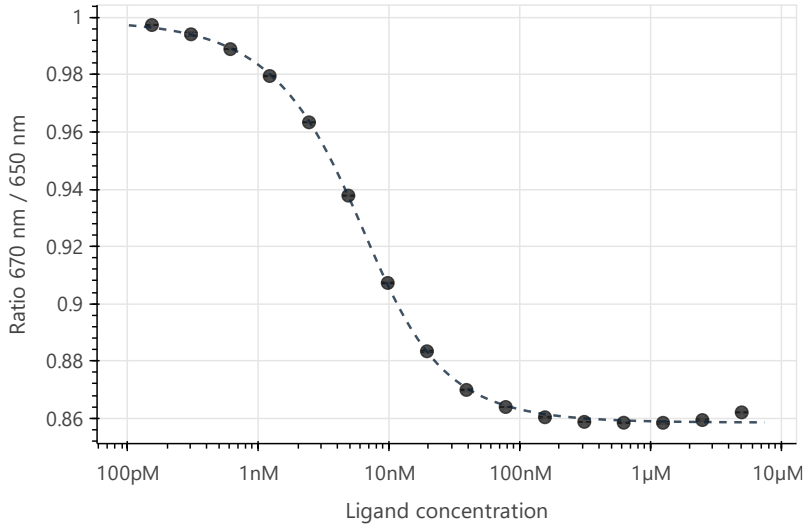
MO.Control v2.6.3 (NanoTemper Technologies GmbH)

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

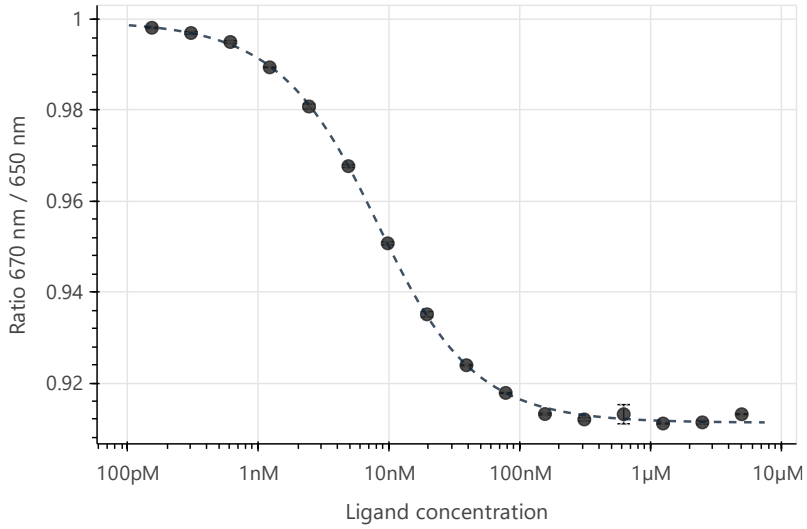
20 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM GSH, 1 mM EDTA, 0.02% Pluronic® F-127, 1% DMSO
10 nM p38-α | 5 µM – 153 pM of inhibitor | 20°C | 100% excitation power

D4. Monolith Results (Dose Response)

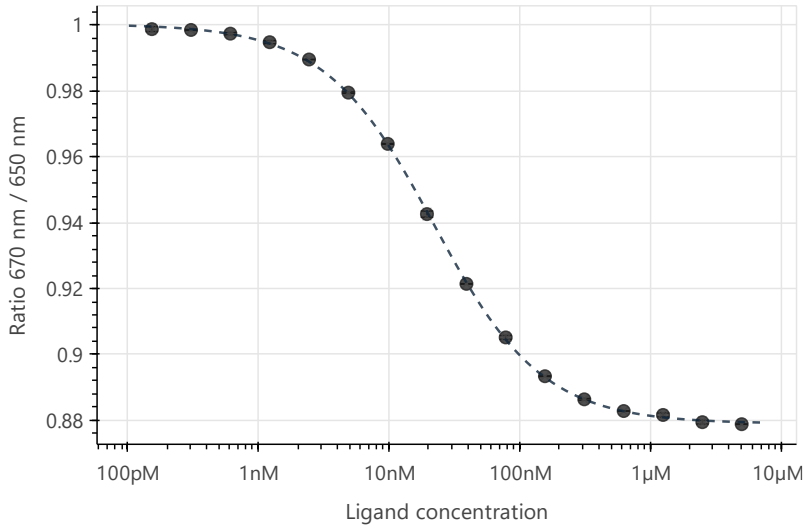
SB 202190 – K_d = 3.21 ± 0.12 nM (S/N = 125.2)



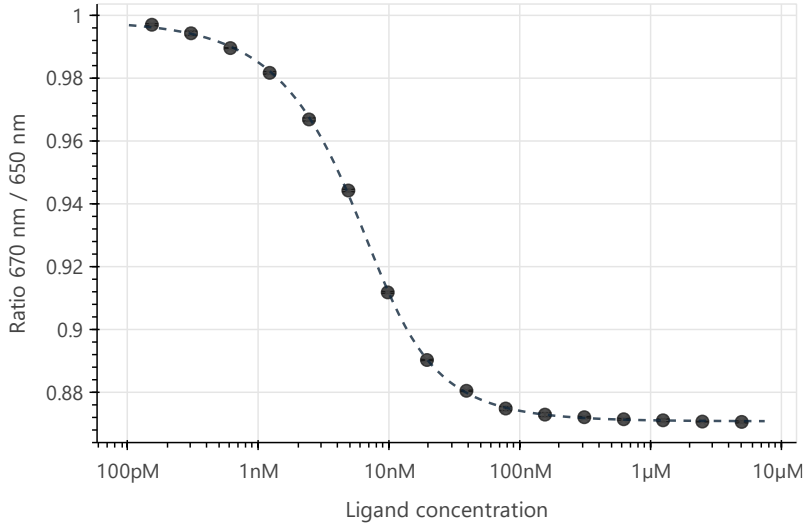
SB 203580 – K_d = 5.94 ± 0.19 nM (S/N = 119.9)



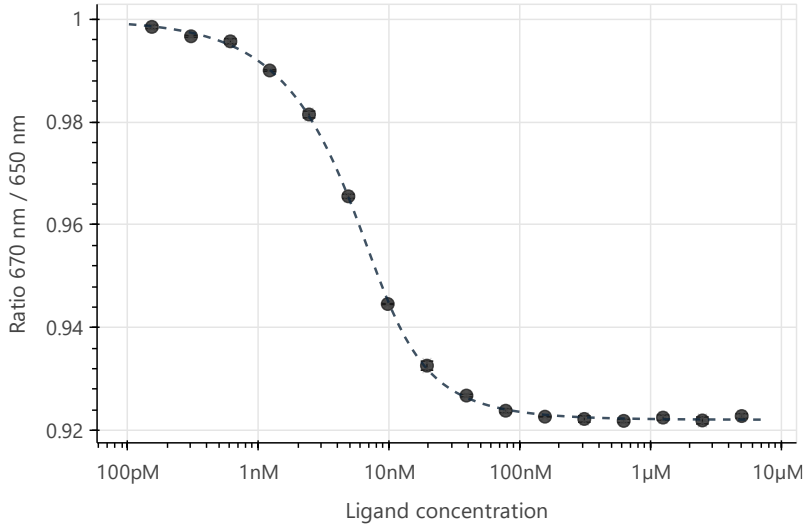
SB 239063 – K_d = 20 ± 0.3 nM (S/N = 237.6)



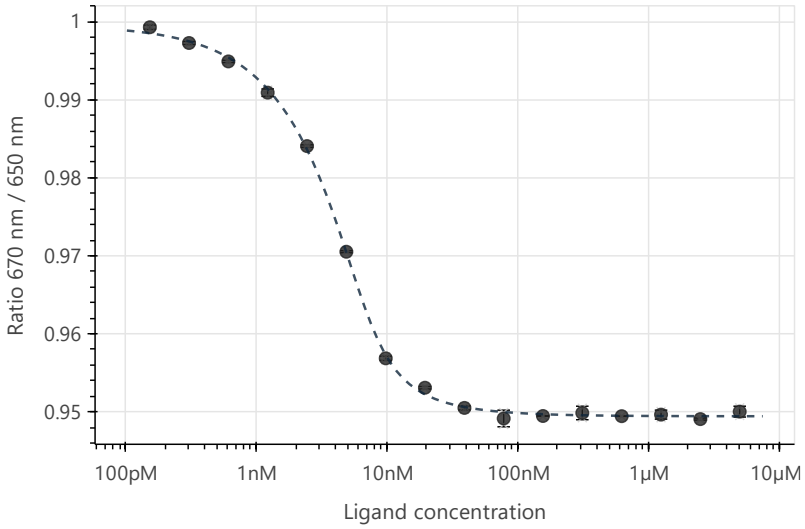
PD 169316 – K_d = 2.51 ± 0.05 nM (S/N = 236.4)



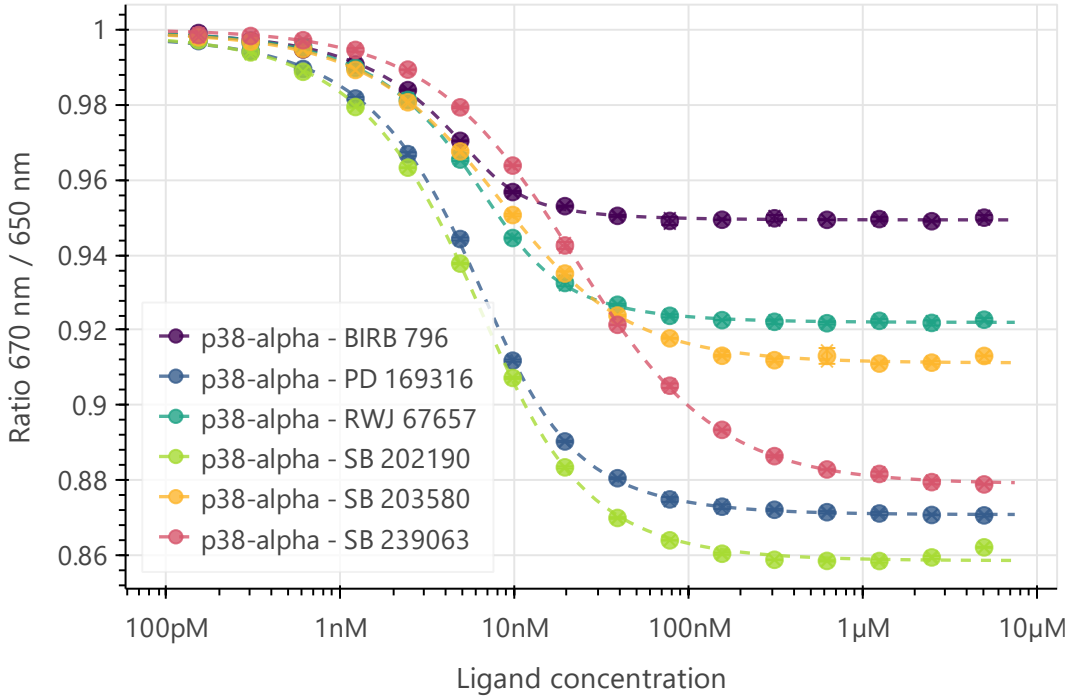
RWJ 67657 – K_d = 1.86 ± 0.07 nM (S/N = 163.9)



BIRB 796 – K_d = 792 ± 61 pM (S/N = 102.8)



Overlay



Ligand	Tycho ΔT_i	Monolith X K_d
SB 202190	+9.2°C	3.21 nM
SB 203580	+8.8°C	5.94 nM
SB 239063	+8.2°C	20 nM
PD 169316	+8.3°C	2.51 nM
RWJ 67657	+12.1°C	1.86 nM
BIRB 796	+20.5°C	792 pM

D5. Reference Results/Supporting Results

SB 202190	K_d = 38 nM	Competitive drug binding assay Frantz et al., Biochemistry, 37 (1998) 13846–13853
SB 203580	K_d = 21 nM	Surface Plasmon Resonance (SPR) Thurmond et al., Eur J Biochem 268 (2001) 5747–5754
SB 239063	IC_{50} = 44 nM	Cell-based assay Underwood et al., Am J Physiol Lung Cell Mol Physiol 279 (2000) 895–902
PD 169316	K_d = 12.5 nM	Surface Plasmon Resonance (SPR) Bio-Rad TechNote 5965
RWJ 67657	K_d = 5 nM	Surface Plasmon Resonance (SPR) Thurmond et al., Eur J Biochem 268 (2001) 5747–5754
BIRB 796	K_d = 0.1 nM	Fluorescence-based assay Regan et al., Bioorganic & Medicinal Chemistry Letters 13 (2003) 3101–3104

E. Contributors

Andreas Langer[2]

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