Monolith Protocol MO-P-002

p38-alpha – SB 203580

Mitogen-activated protein kinase 14 (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 203580 is a specific and potent inhibitor of kinases that binds p38-α with nM 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

p38 mitogen-activated protein kinase (MAP kinase)
Homo sapiens (Human)

A3. Sequence/Formula

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MSQERPTFYR QELNKTIWEV PERYQNLSPV GSGAYGSVCA AFDTKTGLRV AVKKLSRPFQ SIIHAKRTYR ELRLLKHMKH
ENVIGLLDVF TPARSLEEFN DVYLVTHLMG ADLNNIVKCQ KLTDDHVQFL IYQILRGLKY IHSADIIHRD LKPSNLAVNE
DCELKILDFG LARHTDDEMT GYVATRWYRA PEIMLNWMHY NQTVDIWSVG CIMAELLTGR TLFPGTDHID QLKLILRLVG
TPGAELLKKI SSESARNYIQ SLTQMPKMNF ANVFIGANPL AVDLLEKMLV LDSDKRITAA QALAHAYFAQ YHDPDDEPVA
DPYDQSFESR DLLIDEWKSL TYDEVISFVP PPLDQEEMES
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A4. Purification Strategy/Source

Expressed in E. coli BL21, His_6-tagged
Crelux GmbH

A5. Stock Concentration/Stock Buffer

8.82 mg/mL | 197 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

50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% TWEEN® 20

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. Add 95 µL of Labeling Buffer NHS to 5 µL of p38-α (197 µM) to obtain 100 µL of a 10 µM solution.
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 95 µL of Labeling Buffer NHS to obtain 100 µL of a 30 µM dye solution (3x protein concentration).
4. Mix p38-α and dye in a 1:1 volume ratio (200 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 200 µL of the labeling reaction from step 4 to the center of the column and let sample enter the resin bed completely.
9. Add 400 µL of dilution buffer after the sample has entered and discard the flow through.
10. Place column in a new collection tube, add 500 µL of dilution buffer and collect the eluate.
11. Keep the labeled p38-α (~2 µM) on ice in the dark.

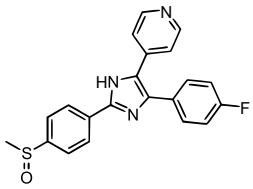
A10. Labeling Efficiency

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

Absorbance A_{280}	0.113	Protein concentration	2.06 µM
Absorbance A_{650}	0.251	Degree-of-labeling (DOL)	0.63

B1. Ligand/Non-Fluorescent Binding Partner

SB 203580



B2. Molecule Class/Organism

p38 MAPK inhibitor

B3. Sequence/Formula

$C_{21}H_{16}FN_3OS$

B4. Purification Strategy/Source

Sigma-Aldrich GmbH
SB307

B5. Stock Concentration/Stock Buffer

1 mg/mL | 2.65 mM
DMSO

B6. Molecular Weight/Extinction Coefficient

377.43 Da

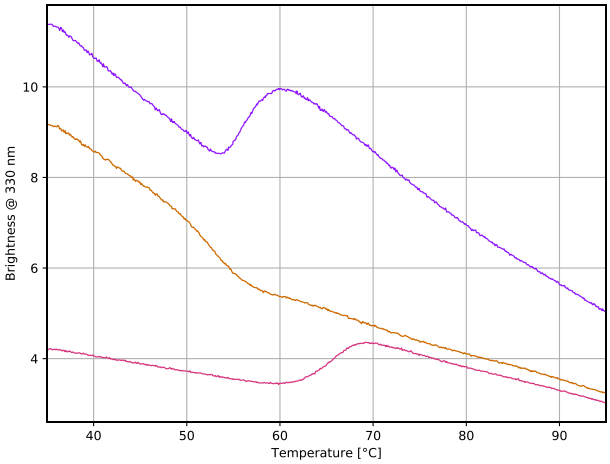
B7. Serial Dilution Preparation

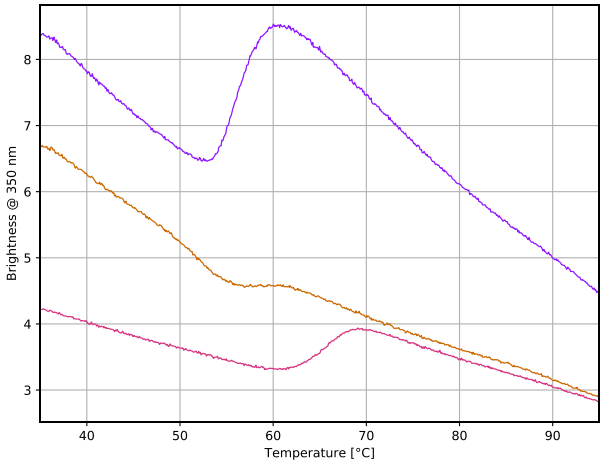
1. Add 8.6 µL of DMSO to 2 µL of the SB 203580 stock to obtain 10.6 µL of a 500 µM solution.
2. Mix 2 µL of the 500 µM SB 203580 solution with 98 µL of dilution buffer to obtain 100 µL of a 10 µM SB 203580 solution.
3. Mix 4 µL of DMSO with 196 µL of dilution buffer to obtain 200 µL of a 2% DMSO solution.
4. Prepare a PCR-rack with 16 PCR tubes. Transfer 20 µL of the 20 µM SB 203580 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 4 µL of labeled p38-α (~2 µM) with 196 µL of dilution buffer to obtain 200 µL of ~40 nM p38-α.
7. Add 10 µL of this solution to each tube from **16** to **1** and mix by pipetting.
8. Incubate for 20 minutes at room temperature in the dark before loading capillaries.

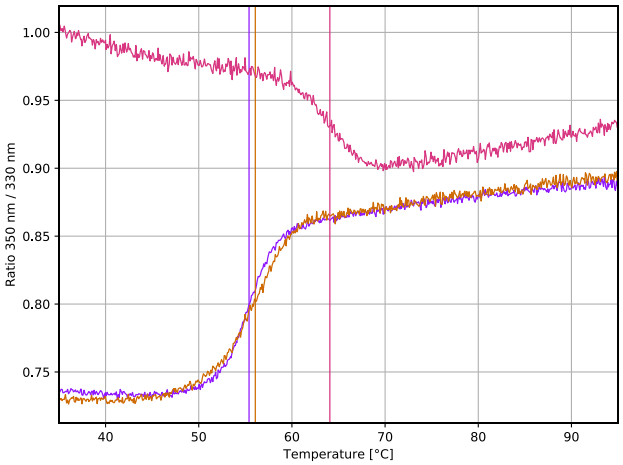
C. Applied Quality Checks

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

Unlabeled	1 µL of 10 µM p38-α + 9 µL of dilution buffer	T_i = 55.4°C
Labeled	5 µL of B-Column eluate (~2 µM) + 5 µL of dilution buffer	T_i = 56.1°C
With ligand	5 µL of B-Column eluate (~2 µM) + 5 µL of 10 µM SB 203580	T_i = 64.1°C







D1. MST System/Capillaries

Monolith NT.115 Red (NanoTemper Technologies GmbH)
Premium Capillaries Monolith NT.115 (MO-K025, NanoTemper Technologies GmbH)

D2. MST Software

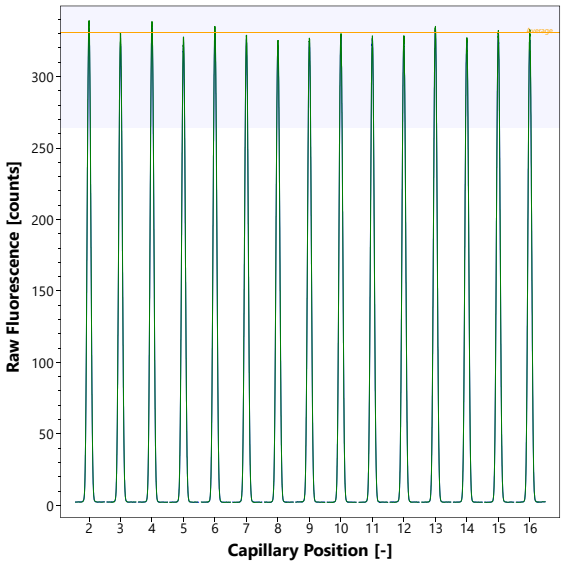
MO.Control v1.6 (NanoTemper Technologies GmbH)
https://nanotempertech.com/monolith-mo-control-software/

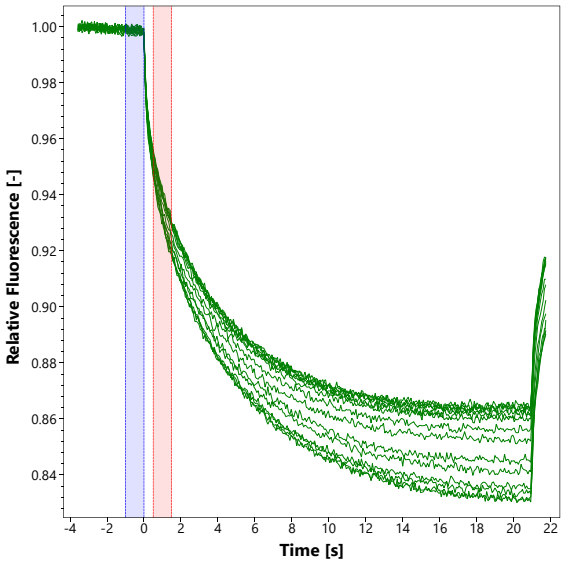
D3. MST Experiment (Assay Buffer/Concentrations/Temperature/MST Power/Excitation Power)

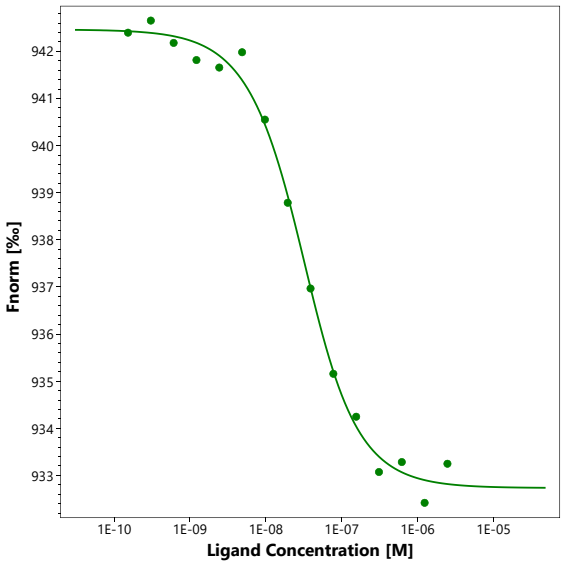
50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% TWEEN® 20, 1% DMSO
20 nM p38-α | 5 µM – 153 pM SB 203580 | 25°C | medium MST power | 40% excitation power

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

K_d = 21.6 nM







D5. Reference Results/Supporting Results

K_d = 21 nM	Surface Plasmon Resonance (SPR) Thurmond et al., Eur J Biochem 268 (2001) 5747–5754
K_d = 15 nM	Isothermal Titration Calorimetry (ITC) Young et al., J Biol Chem 272 (1997) 12116-12121

E. Contributors

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