Monolith Protocol MO-P-004

p38-alpha – SB 203580 (purification-free)

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. Using His-tag labeling, the interaction can be measured purification-free in cell lysate.

protein – small molecule interaction | kinase | inhibitor | His-tag | cell lysate | purification-free

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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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Constructs: p38-α-mNeonGreen-His_6 | mNeonGreen-His_6 (negative control)

A4. Purification Strategy/Source

Cloning

Using In-Fusion Cloning technology (Takara Bio USA, Inc.), p38-α coding sequence was cloned in a pcDNA3.1 mammalian expression vector behind a CMV promotor and separated from the mNeongreen-His_6-tag by the linker sequence ESGSGS. A pcDNA3.1 vector coding for only mNeongreen-His_6 was used as a control.

Expression

The two plasmids expressing mNeongreen-His_6 with and without the p38-α sequence were transfected into 3 × 10^6 HeLa cells using separate T-75 flasks. Cells were grown for 24 h reaching approximately 10^7 cells.

Lysate Preparation

Cells were pelleted by centrifugation and resuspended in 1 mL PBS-T buffer[1] (137 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4, 0.05% TWEEN® 20), supplemented with protease inhibitors. At this step, the cells were disrupted using a Dounce homogenizer and centrifuged again at 14 000 × g for 30 min at 4°C to remove cell debris. Obtained supernatant was diluted 1:10 in PBS-T and supplemented with protease inhibitors (PMSF)[2].

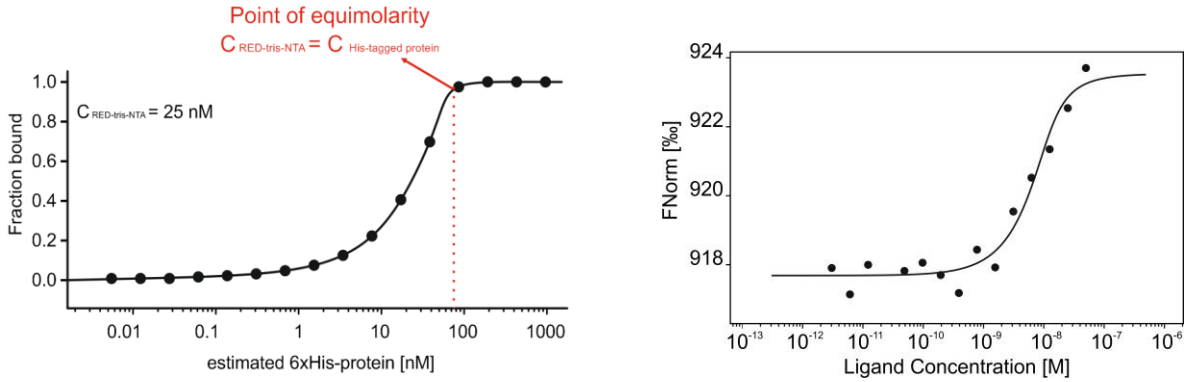
[1] RIPA buffer was tested but did not work for this assay.
[2] It is recommended to work with freshly prepared cell lysates when performing MST experiments.

Concentration Determination

Concentration of His_6-tagged p38-α in cell lysate was determined as described below. A 16-point serial dilution of cell lysate expressing p38-α was prepared using non-transfected HeLa cell lysates in 10 µL of final volume. 10 µL of 50 nM RED-tris-NTA dye were added to each reaction tube, resulting in a final dye concentration of 25 nM. The reaction mixture was incubated for 30 min at room temperature and loaded into Premium Capillaries Monolith NT.115 (MO-K025, NanoTemper Technologies GmbH).

The MST experiment was carried out using 50% Excitation and medium MST power. MST data were evaluated after 10 s MST-on time. Since the affinity of the RED-tris-NTA dye towards the His_6-tag is in the low nM range (2.1 nM as measured for the purified protein) and therefore well below the concentration of RED-tris-NTA, the binding curve displays a 'kink' at the point where equimolar concentrations of dye and His-tagged protein are present (simulated dose response, left panel).

As there is a single binding site for the RED-tris-NTA dye, one can assume that dye and protein concentrations are equivalent at the 'kink', so that the concentration of the protein at the start of the dilution can be calculated. In this experiment, saturation of the binding curve was not reached (right panel) suggesting that the concentration of the protein in the lysate was less than 2-fold above the concentration of the dye (less or equal to 50 nM).



A5. Stock Concentration/Stock Buffer

50 nM (p38-α-mNeonGreen-His_6)
Phosphate-buffered saline (PBS, pH 7.4), 2 mM PMSF, 0.05% TWEEN® 20

A6. Molecular Weight/Extinction Coefficient

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

A7. Dilution Buffer

Phosphate-buffered saline (PBS, pH 7.4), 0.05% TWEEN® 20

A8. Labeling Strategy

Monolith His-Tag Labeling Kit RED-tris-NTA (MO-L008, NanoTemper Technologies GmbH)
1* PBS-T | 1* 250 pmol RED-tris-NTA Dye

A9. Labeling Procedure

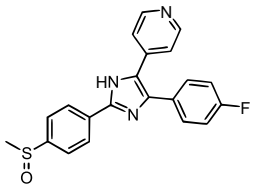
1. Prepare a 100 nM dye solution by mixing 2 µL of dye (5 µM) and 98 µL PBS-T.
2. Add 100 µL of lysate containing p38α-mNeonGreen-His_6.
3. Incubate for 30 minutes at room temperature in the dark.
4. Centrifuge the reaction mixture for 10 min at 15,000 × g.

A10. Labeling Efficiency

N/A

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. Mix 2 µL of the 2.65 mM SB 203580 solution with 98 µL of dilution buffer to obtain 100 µL of a 53 µM SB 203580 solution in dilution buffer containing 2% DMSO.
2. Mix 4 µL of DMSO with 196 µL of dilution buffer to obtain 200 µL of a 2% DMSO solution.
3. Prepare a PCR-rack with 16 PCR tubes. Transfer 20 µL of the 53 µM SB 203580 solution into tube **1**. Then, transfer 10 µL of the 2% DMSO solution into tubes **2** to **16**.
4. 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.
5. Add 10 µL of labeled p38α-mNeonGreen-His_6 to each tube from **16** to **1** and mix by pipetting.
6. Incubate for 20 minutes in the dark and then centrifuge for 10 min at 14000 × g before loading capillaries.

D1. MST System/Capillaries

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

D2. MST Software

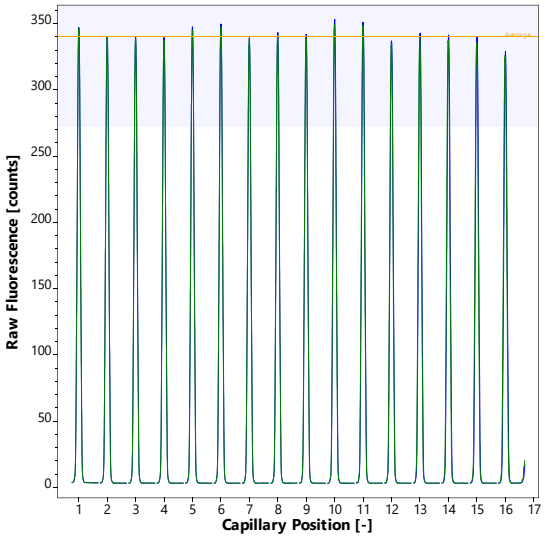
MO.Control v1.6 (NanoTemper Technologies GmbH)
nanotempertech.com/monolith-mo-control-software

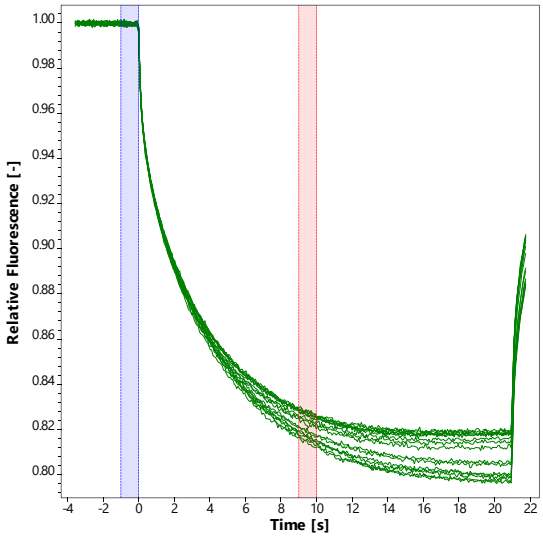
D3. MST Experiment (Assay Buffer/Concentrations/Temperature/MST Power/Excitation Power)

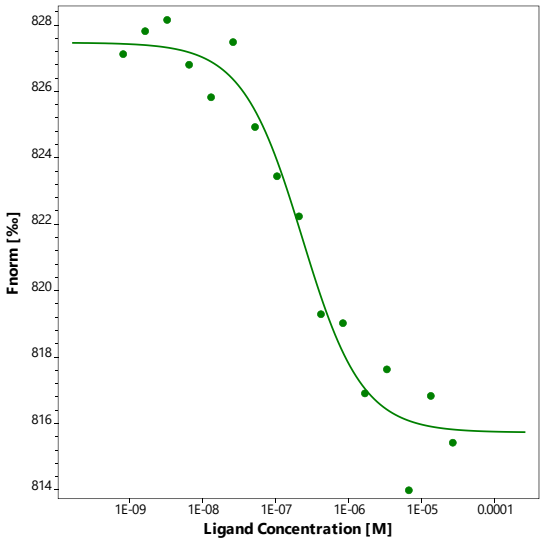
Phosphate-buffered saline (PBS, pH 7.4), 0.05% TWEEN® 20, 1% DMSO
25 nM p38-α | 26.5 µM – 809 pM SB 203580 | 25°C | high MST power | 20% excitation power

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

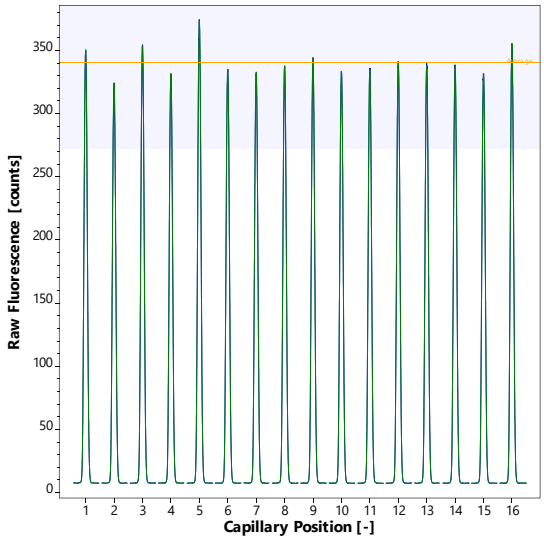
K_d = 116 nM (p38-α-mNeonGreen-His_6 – SB 203580)

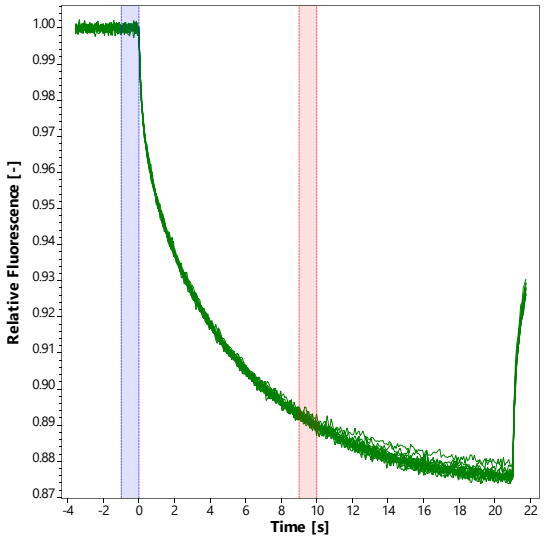


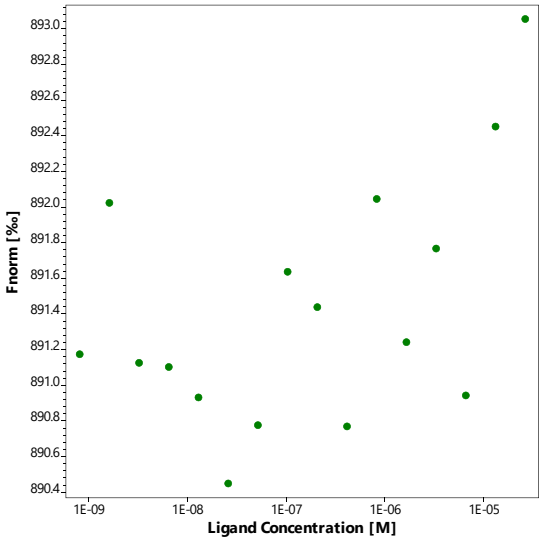




K_d = N/A (mNeonGreen-His_6 – SB 203580, negative control)







D5. Reference Results/Supporting Results

K_d = 116 nM Microscale Thermophoresis
Bortoschik et al., Scientific Reports 8 (2018) 4977

E. Contributors

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