Monolith Protocol MO-P-003

p38-alpha – SB 203580 (competitive assay)

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. Compounds that bind to the ATP site or to an allosteric site altering the conformation of the ATP site (Type I and Type II kinase inhibitors) can be detected in a competitive assay using a fluorescent tool compound that is based on ATP-competitive kinase inhibitors.

protein – small molecule interaction | kinase | inhibitor | competitive assay

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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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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

Competitive binding assay with Alexa Fluor™ 647 labeled Kinase Tracer 199 (T-199)
Thermo Fisher Scientific
PV5830

Alexa Fluor™ 647 labeled Kinase Tracer 199 is based on ATP-competitive kinase inhibitors which makes it suitable for detection of any compounds that bind to the ATP site or to an allosteric site altering the conformation of the ATP site (Type I and Type II kinase inhibitors).

A9. Labeling Procedure

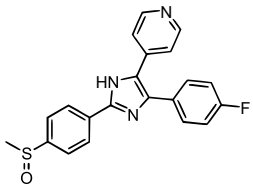
N/A

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
S8307

B5. Stock Concentration/Stock Buffer

1 mg/mL | 2.65 mM
DMSO

B6. Molecular Weight/Extinction Coefficient

377.43 Da

B7. Serial Dilution Preparation

Direct binding assay

1. Add 2 µL of 197 µM p38-α to 195 µL of dilution buffer to obtain 197 µL of a 2 µM p38-α solution.
2. Prepare a PCR-rack with 16 PCR tubes. Transfer 20 µL of 2 µM p38-α into tube **1**. Then, transfer 10 µL of dilution buffer into tubes **2** to **16**.
3. 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.
4. Add 225 µL of DMSO to 25 µL of 25 µM T-199 to obtain 250 µL of a 2.5 µM solution. Prepare aliquots of 4 µL and store at -20°C.
5. Add 196 µL of dilution buffer to 4 µL of 2.5 µM T-199 to obtain 200 µL of a 50 nM T-199 solution.
6. Add 10 µL of T-199 (50 nM) to each tube from **16** to **1** and mix by pipetting.
7. Incubate for 20 minutes at room temperature in the dark before loading capillaries.

Competitive binding assay

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 10 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 T-199 (2.5 µM) and 5 µL of p38-α (2 µM) with 191 µL of dilution buffer to obtain 200 µL of a 50 nM T-199, 50 nM p38-α solution.[1]
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.

D1. MST System/Capillaries

Monolith NT.115 Red (NanoTemper Technologies GmbH)
Capillaries Monolith NT.115 (MO-K022, NanoTemper Technologies GmbH)

D2. MST Software

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

[1] As the K_d between T-199 and p38-α is 15.5 nM (c.f. section D4), a final concentration of 25 nM p38-α is sufficient for complex formation, but still low enough for accurate K_d determination of the SB 203580 – p38-α interaction.

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

Direct binding assay

50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% TWEEN® 20, 1% DMSO
25 nM T-199 | 1 µM – 31 pM p38-α | 25°C | low MST power | 20% excitation power

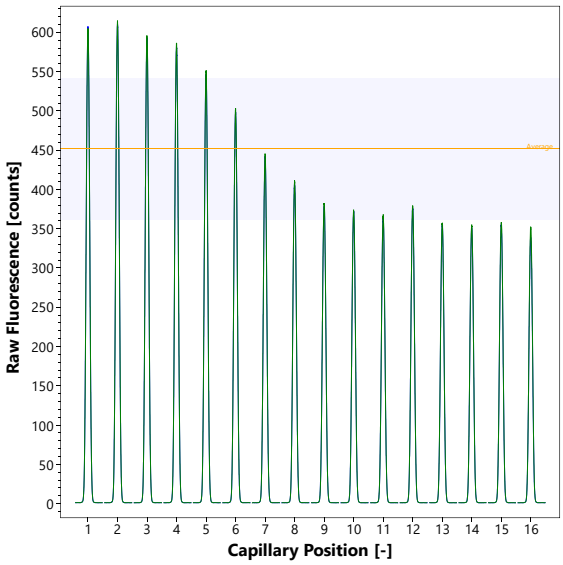
Competitive binding assay

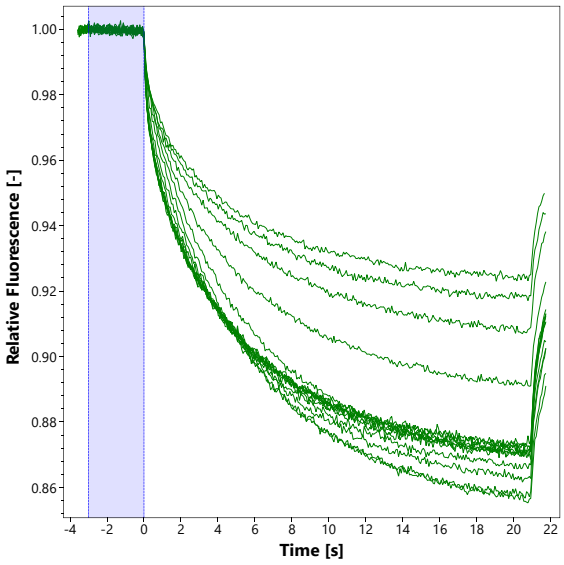
50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% TWEEN® 20, 1% DMSO
25 nM T-199, 25 nM p38-α | 5 µM – 153 pM SB 203580 | 25°C | low MST power | 20% excitation power

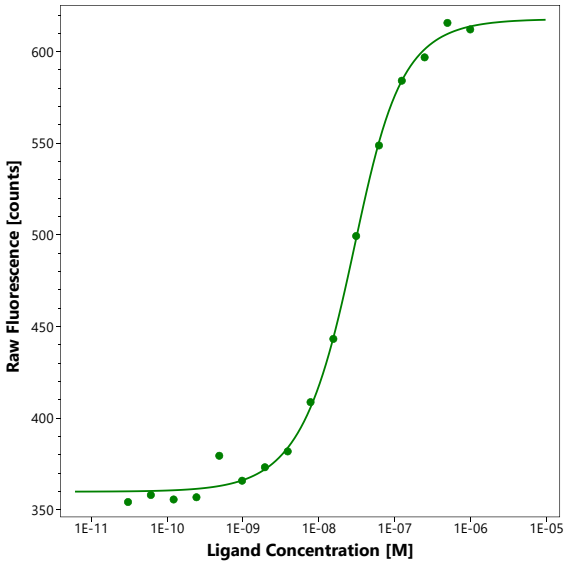
D4. MST Results (Capillary Scan/Time Traces/Dose Response)

Direct binding assay

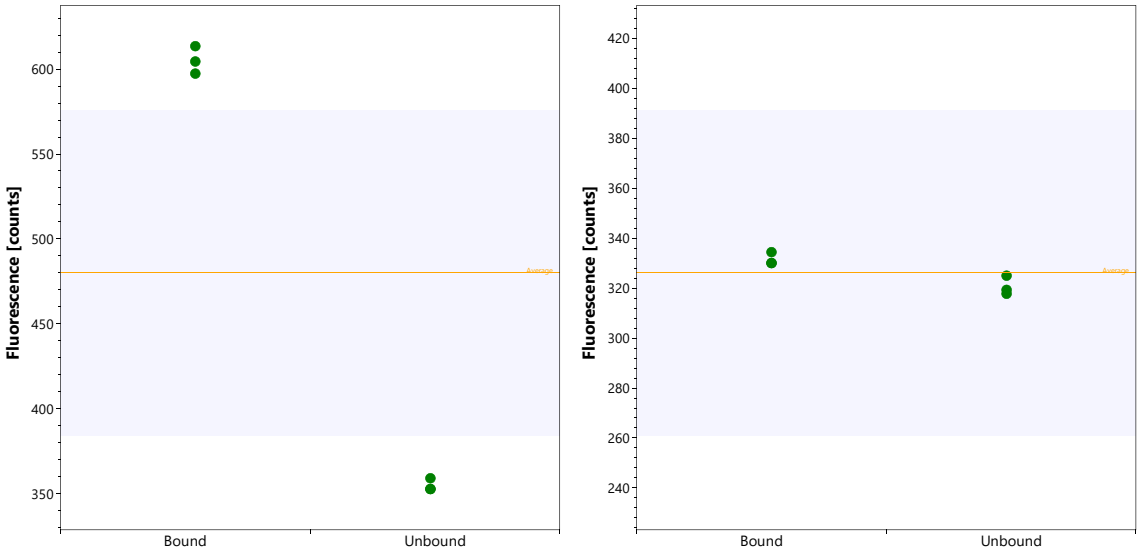
K_d = 15.5 nM (T-199 – p38-α)





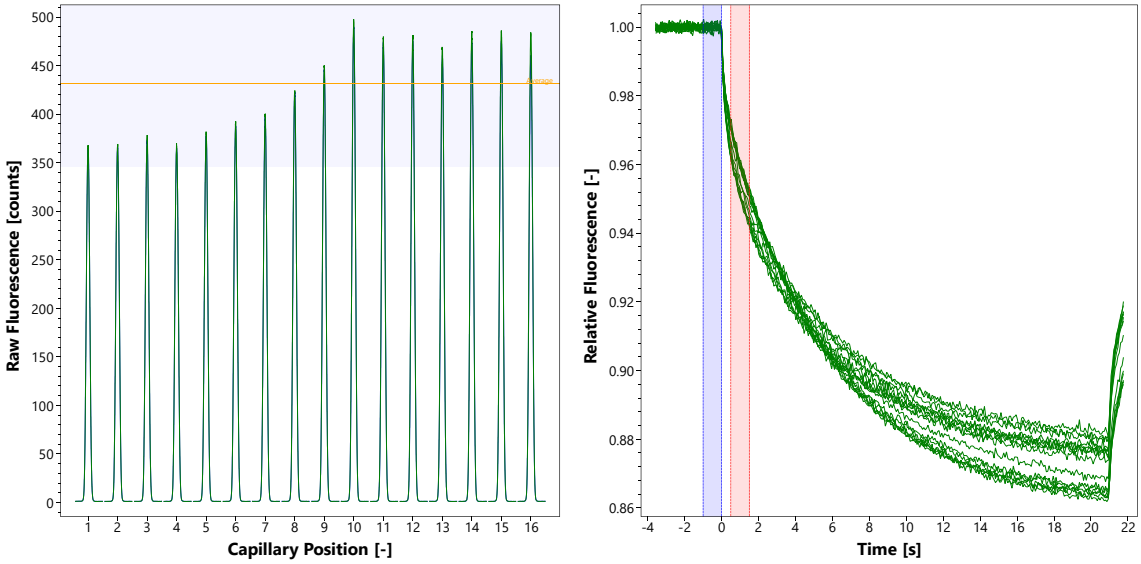


SD Test[2]:

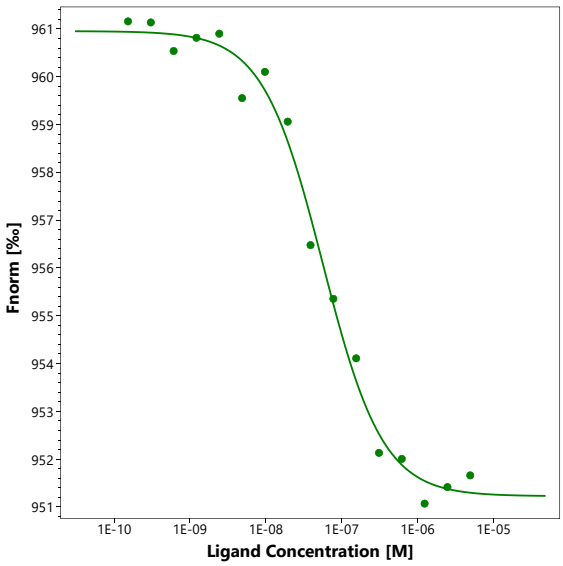


Competitive binding assay

EC_{50} = 56.7 nM | K_i = 13.6 nM[3] (p38-α – SB 203580)



[2] Do **not** centrifuge tubes.
[3] For calculation of K_i, see also the NanoTemper 'FAQ Competitive Binding Assay'.



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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