Monolith Protocol MO-P-028

C3d – ZINC72165106

Complement C3d is a cleavage product formed following activation of the complement cascade. The fragment covalently attaches to cell surfaces via a thioester, making it a long-lived biomarker of complement-mediated inflammation and tissue damage. ZINC72165106 is a small molecule discovered through virtual high-throughput screening capable of binding to C3d.

protein – small molecule interaction | complement

A1. Target/Fluorescent Molecule

C3d
uniprot.org/uniprot/P01024

A2. Molecule Class/Organism

Complement protein
Homo sapiens (Human)

A3. Sequence/Formula

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HLIVTPSGCG EQNMIGMTPT VIAVHYLDET EQWEKFGLEK RQGALELIKK GYTQQLAFRQ PSSAFAAFVK RAPSTWLTAY
VVKVFSLAVN LIAIDSQVLC GAVKWLILEK QKPDGVFQED APVIHQEMIG GLRNNNEKDM ALTAFVLISL QEAKDICEEQ
VNSLPGSITK AGDFLEANYM NLQRSYTVAI AGYALAQMGR LKGPLLNKFL TTAKDKNRWE DPGKQLYNVE ATSYALLALL
QLKDFDFVPP VVRWLNEQRY YGGGYGSTQA TFMVFQALAQ YQKDAPDHQE LNLDVSLQLP SR
```

A4. Purification Strategy/Source

Precursor protein (C3) purified from plasma and cleaved using established methods (see manufacturer website)
Complement Technology
A117

A5. Stock Concentration/Stock Buffer

1 mg/mL | 29.6 µM
Phosphate-buffered saline (PBS), pH 7.2

A6. Molecular Weight/Extinction Coefficient

33.8 kDa
45,505 $M^{-1}cm^{-1}$ (ε_{280})

A7. Dilution Buffer

Phosphate buffered saline (PBS), pH 7.4, 0.05% TWEEN® 20, 0.1% PEG 8000

A8. Labeling Strategy

Monolith Protein Labeling Kit RED – NHS 2nd Generation (MO-L011, NanoTemper Technologies GmbH)
1* Labeling Buffer NHS | 1* A-Column | 1* Dye RED-NHS 2nd Generation (10 µg) | 1* B-Column

A9. Labeling Procedure

1. Add 49.3 µL of Labeling Buffer NHS to 50.7 µL of 29.6 µM C3d to obtain 100 µL of a 15 µM solution.
2. Use the A-Column to perform a buffer exchange into Labeling Buffer NHS.
   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 1.5 mL microcentrifuge collection tube.
   c. Centrifuge at **1500 × g** for **1 min** to remove excess liquid.
   d. Add 300 µL of Labeling Buffer NHS and centrifuge at **1500 × g** for **1 min** (3x).
   e. Place 100 µL of the 15 µM C3d 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 100 µL of ~10 µM C3d (70% recovery).
3. 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.
4. Mix 7.5 µL of the 600 µM dye solution with 92.5 µL of Labeling Buffer NHS to obtain 100 µL of a 45 µM dye solution (4.5x protein concentration).
5. Mix C3d and dye in a 1:1 volume ratio (200 µL final volume, 3.75% 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 200 µL of the labeling reaction from step 5 to the center of the column and let sample enter the bed completely.
10. Add 300 µL of dilution buffer after the sample has entered and discard the flow through.
11. Add 600 µL of dilution buffer and collect the eluate three drops at a time (~100 µl fractions) in 0.6 mL tubes.
12. Measure OD_{280} and OD_{650} of each fraction, then combine fractions containing protein (3 and 4).
13. Centrifuge labeled C3d at 15,000 × g for 10 minutes at 4°C, then collect supernatant. Measure OD_{280} and OD_{650} (yields ~200 µL of 2.7 µM C3d), then aliquot and store at -80°C.

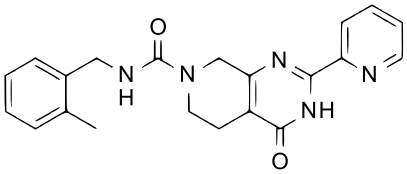
A10. Labeling Efficiency

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

Absorbance A_{280}	0.16	Protein concentration	2.7 µM
Absorbance A_{650}	0.96	Degree-of-labeling (DOL)	1.8

B1. Ligand/Non-Fluorescent Binding Partner

ZINC72165106



N-(2-methylbenzyl)-4-oxo-2-pyridin-2-yl-4,5,6,8-tetrahydropyrido[3,4-d]pyrimidine-7(3H)-carboxamide

B2. Molecular Class/Organism

Small molecule

B3. Sequence/Formula

$C_{21}H_{21}N_5O_2$

B4. Purification Strategy/Source

ChemBridge Corporation
56710071

B5. Stock Concentration/Stock Buffer

Dry film (5 mg)

B6. Molecular Weight/Extinction Coefficient

375.40 Da

B7. Serial Dilution Preparation

1. Resuspend dry film in 266 µL of DMSO to obtain a 50 mM ligand solution. Centrifuge stock solution at 15,000 × g for 10 minutes at 25°C.
2. Dissolve 2.5 µL of the 50 mM ligand stock in 97.5 µL DMSO to obtain 100 µL of a 1250 µM ligand solution in 100% DMSO.
3. Prepare a PCR rack with 16 PCR tubes. Transfer 20 µL of the 1250 µM ligand solution into tube **1**. Then, add 10 µL DMSO to 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 48 µL of dilution buffer to 16 new PCR tubes. Transfer 2 µL of each ligand dilution into the corresponding tube in the new set to obtain 50 µL of the ligand dilution series in dilution buffer containing 4% DMSO.
6. Prepare a final set of 16 PCR tubes. Mix each ligand dilution (in 4% DMSO) well by pipetting and transfer 10 µL into the corresponding tube in the final set.
7. Mix 2 µL of labeled C3d with 458 µL of dilution buffer to obtain 460 µL of ~12 nM C3d.
8. Add 10 µL of labeled C3d (~12 nM) to each tube from **16** to **1** and mix by pipetting.
9. Incubate for 15 minutes at room temperature in the dark before loading capillaries.

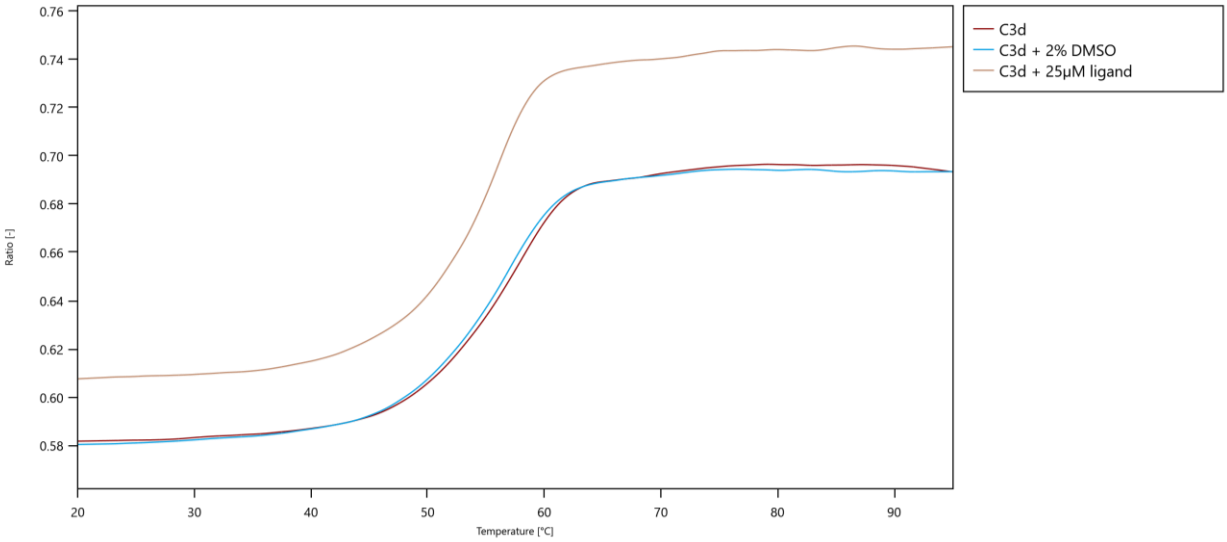
B8. SD-Test

1. Prepare the SD-mix: Dilute 400 µL of 10% SDS and 40 µL of 1 M DTT in 560 µL water to obtain a solution containing 4% SDS and 40 mM DTT.
2. Transfer 7 µL of the SD-mix to six PCR tubes.
3. Centrifuge the remainder of tubes **1** to **3** and **14** to **16** from the 1:1 serial dilution above for 10 minutes at 15,000 x g at 4°C.
4. Carefully collect 7 µL supernatant from each tube and add to tubes containing 7 µL SD-mix. Mix well by pipetting.
5. Place samples on a heat block set to 95°C for 5 minutes to denature the protein, then allow to cool at 25°C for 10 minutes before loading into capillaries.

C. Applied Quality Checks

Validation of structural integrity and functionality of labeled C3d using Prometheus NT.48:
nanotempertech.com/prometheus

C3d	1 µM labeled C3d in dilution buffer	57.3°C
C3d + 2% DMSO	1 µM labeled C3d in dilution buffer + 2% DMSO	56.6°C
C3d + 25 µM ligand	1 µM labeled C3d in dilution buffer + 25 µM ligand (2% DMSO final)	55.6°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

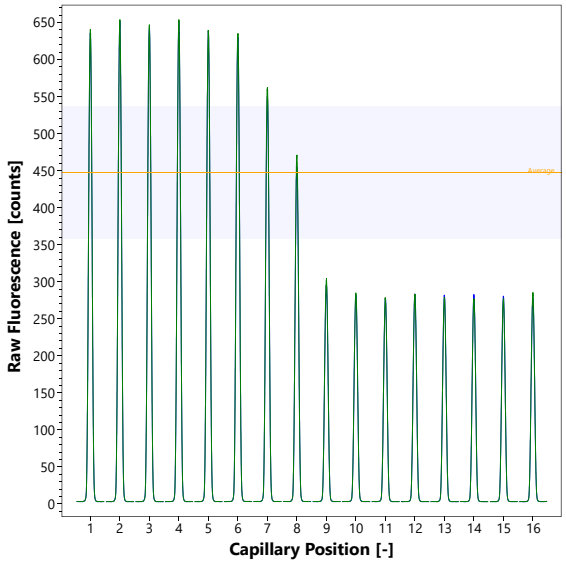
MO.Control v1.6 (NanoTemper Technologies GmbH)
nanotempertech.com/monolith-mo-control-software

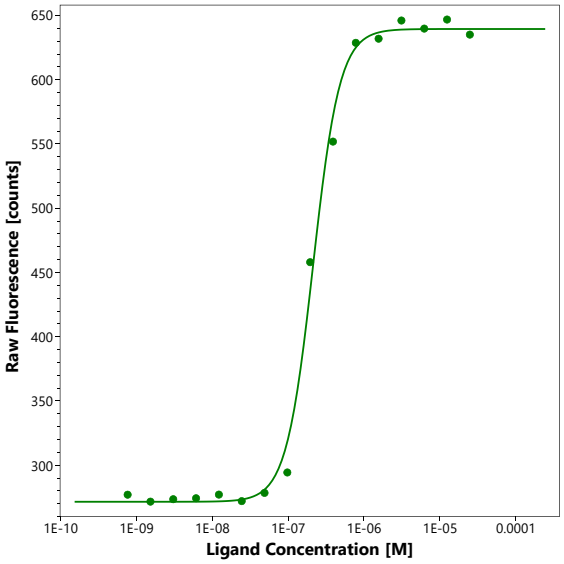
D3. MST Experiment (Assay Buffer/Concentrations/Temperature/MST Power/Excitation Power)

Phosphate-buffered saline (PBS), pH 7.4, 0.05% TWEEN® 20, 0.1% PEG 8000, 2% DMSO
6 nM C3d | 25 µM – 763 pM ZINC72165106 | 25°C | high MST power | 80% excitation power

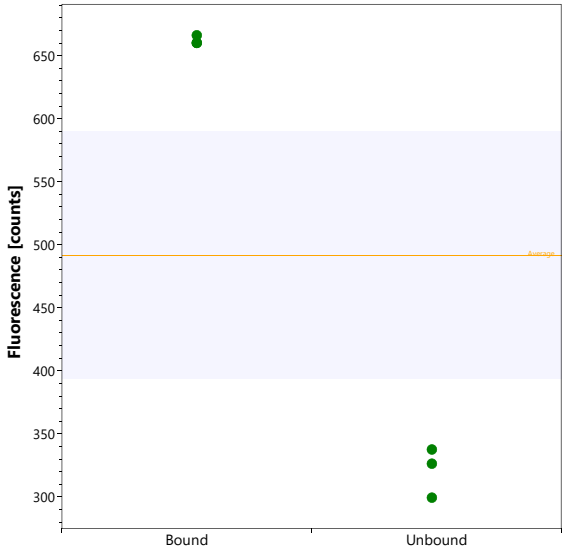
D4. MST Results (Capillary Scan/Time Traces/Dose Response)

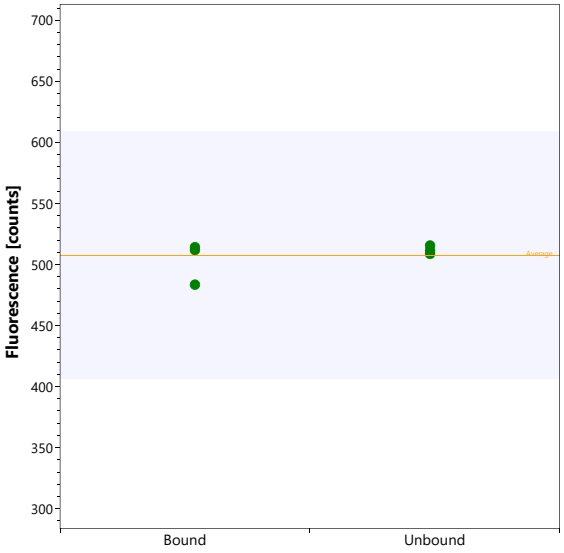
EC_{50} = 214 nM Initial Fluorescence Analysis





SD-Test[1]:





[1] Due to the ligand-dependent fluorescence changes, an SD-test was performed to confirm binding-dependent change. As the changes in initial fluorescence were concluded to be binding-specific, the initial fluorescence data was used for binding curve fit and EC_{50} determination.

D5. Reference Results/Supporting Results

K_d = 1.7 µM Microscale Thermophoresis (MST)
Gorham et al., J. Med. Chem. 58, 9535-9545 (2015)

E. Contributors

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