Monolith Protocol MO-P-078

Carbonic Anhydrase II – Furosemide (label-free)

Bovine carbonic anhydrase II catalyzes the rapid interconversion of carbon dioxide and water to bicarbonate and protons. Its active site contains a zinc ion. Furosemide is a potent carbonic anhydrase inhibitor.

protein – small molecule interaction | inhibitor | label-free

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A1. Target/Fluorescent Molecule

Carbonic Anhydrase Isozyme II from bovine erythrocytes (bCA-II)
uniprot.org/uniprot/P00921

A2. Molecule Class/Organism

Carbonic anhydrase
Bos taurus (Bovine)

A3. Sequence/Formula

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MSHHWGYGKH NGPEHWHKDF PIANGERQSP VDIDTKAVVQ DPALKPLALV YGEATSRRMV NNGHSFNVEY DDSQDKAVLK
DGPLTGTYRL VQFHFHWGSS DDQGSEHTVD RKKYAAELHL VHWNTKYGDF GTAAQQPDGL AVVGVFLKVG DANPALQKVL
DALDSIKTKG KSTDFPNFDP GSLLPNVLDY WTYPGSLTTP PLLESVTWIV LKEPISVSSQ QMLKFRTLNF NAEGEPELLM
LANWRPAQPL KNRQVRGFPK
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A4. Purification Strategy/Source

Sigma-Aldrich GmbH
C2522

A5. Stock Concentration/Stock Buffer

5 mg lyophilized powder

A6. Molecular Weight/Extinction Coefficient

29.1 kDa
50,420 $M^{-1}cm^{-1}$ (ε_{280})

A7. Dilution Buffer

50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 0.1% Pluronic® F-127

A8. Labeling Strategy

Trp and Tyr fluorescence

A9. Labeling Procedure

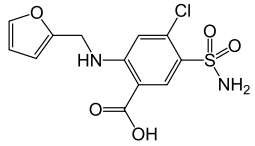
1. Dissolve ~200 µg of bCA-II in 100 µL of dilution buffer (approx. ~70 µM concentration).
2. Centrifuge the tube at 15,000 rpm at 4°C for 20 minutes to remove aggregates. Then, carefully transfer 95 µL of the supernatant of the tube into a new tube and mix well by pipetting.
3. Determine the concentration spectroscopically using an extinction coefficient of 50,420 $M^{-1}cm^{-1}$ (ε_{280}). Afterwards, adjust the concentration to 40 µM with dilution buffer.

A10. Labeling Efficiency

N/A

B1. Ligand/Non-Fluorescent Binding Partner

Furosemide



B2. Molecule Class/Organism

Carbonic anhydrase inhibitor

B3. Sequence/Formula

$C_{12}H_{11}ClN_2O_5S$

B4. Purification Strategy/Source

Sigma-Aldrich GmbH
F4381

B5. Stock Concentration/Stock Buffer

33 mg/mL | 100 mM
DMSO

B6. Molecular Weight/Extinction Coefficient

330.74 Da

B7. Serial Dilution Preparation

1. Dissolve 33 mg of furosemide in 1 mL of DMSO to obtain a 100 mM furosemide solution.
2. Add 2 µL of 100 mM acetazolamide to 18 µL of DMSO to obtain 20 µL of a 10 mM solution.
3. Mix 5 µL of the 1 mM acetazolamide solution with 495 µL of dilution buffer to obtain 500 µL of a 100 µM furosemide solution.
4. Mix 10 µL of DMSO with 990 µL of dilution buffer to obtain 1 mL of a 1% DMSO solution.
5. Prepare a PCR-rack with 16 PCR tubes. Transfer 40 µL of the 100 µM furosemide solution into tube **1**. Then, transfer 20 µL of the 1% DMSO solution into tubes **2** to **16**.
6. Prepare a 1:1 serial dilution by transferring 20 µL from tube to tube. Mix carefully by pipetting up and down. Remember to discard 20 µL from tube **16** to get an equal volume of 20 µL for all samples.
7. Mix 4 µL of bCA-II (40 µM) with 396 µL of dilution buffer to obtain 400 µL of 400 nM bCA-II.
8. Add 20 µL of bCA-II (400 nM) to each tube from **16** to **1** and mix by pipetting.
9. Incubate tubes for 5 minutes in the dark before loading capillaries.

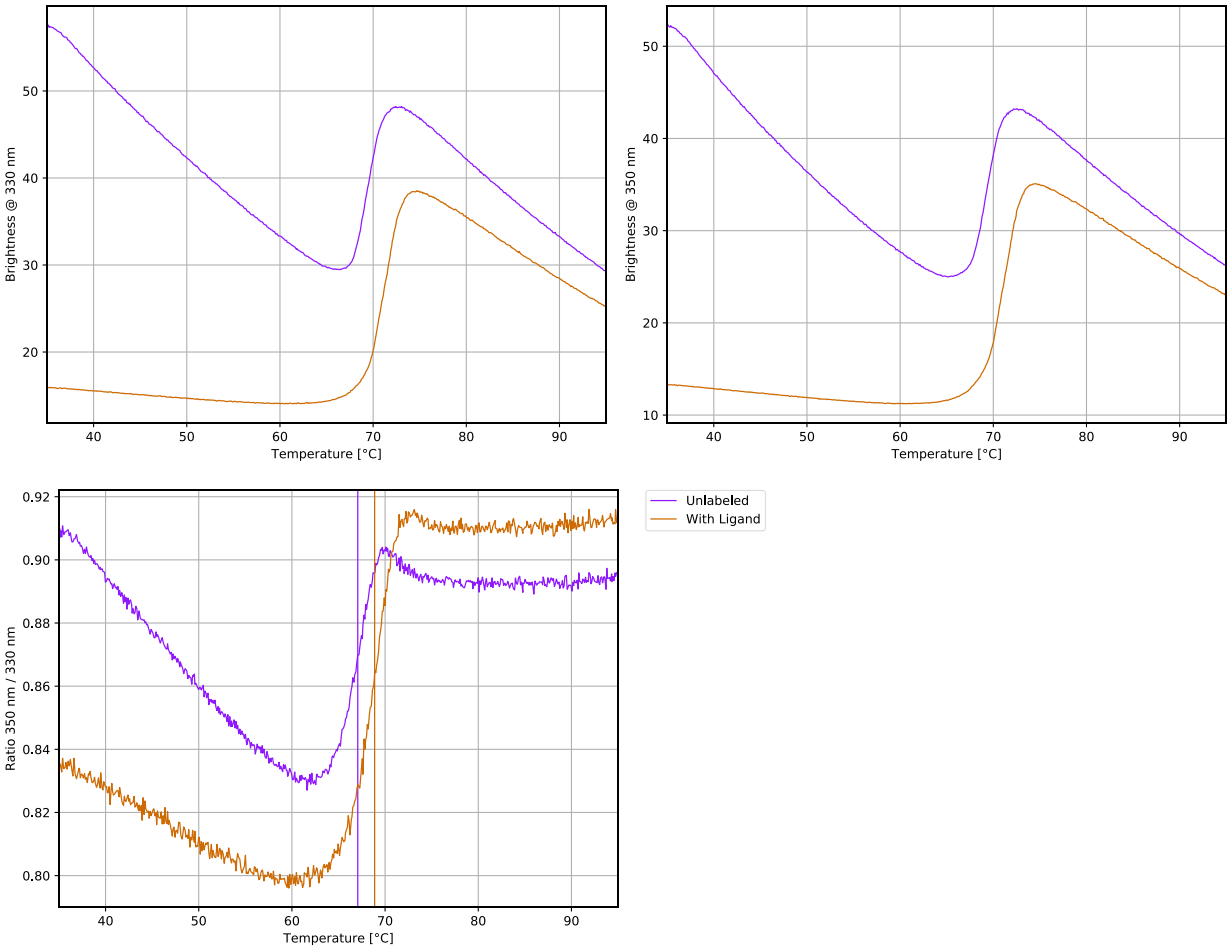
B8. SD-Test

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. Add 7 µL from the remainder of tubes **1** to **3** and **14** to **16** to the tubes containing 7 µL SD-mix. Mix well by pipetting.
4. 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 bCA-II using Tycho NT.6:
nanotempertech.com/tycho

Unlabeled	5 µL of bCA-II (10 µM) + 5 µL of dilution buffer containing 1% DMSO	T_i = 67.1°C
With ligand	5 µL of bCA-II (10 µM) + 5 µL of 100 µM furosemide	T_i = 68.9°C



D1. MST System/Capillaries

Monolith Label-Free (NanoTemper Technologies GmbH)
Premium Capillaries Monolith NT.LabelFree (MO-Z025, NanoTemper Technologies GmbH)

D2. MST Software

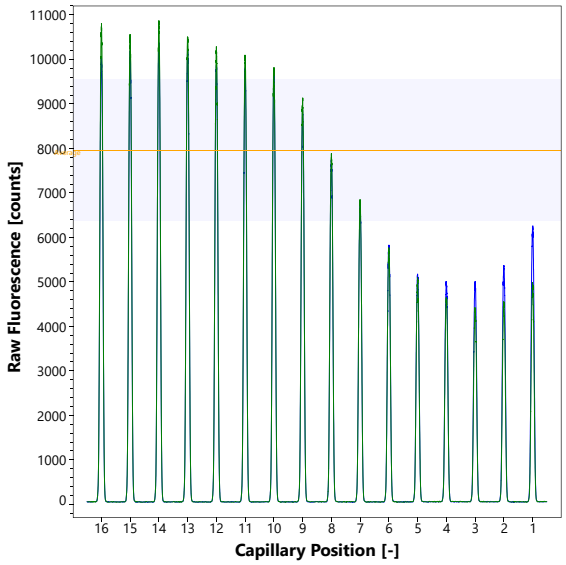
MO.Control v2.0.4 (NanoTemper Technologies GmbH)
nanotempertech.com/monolith-mo-control-software

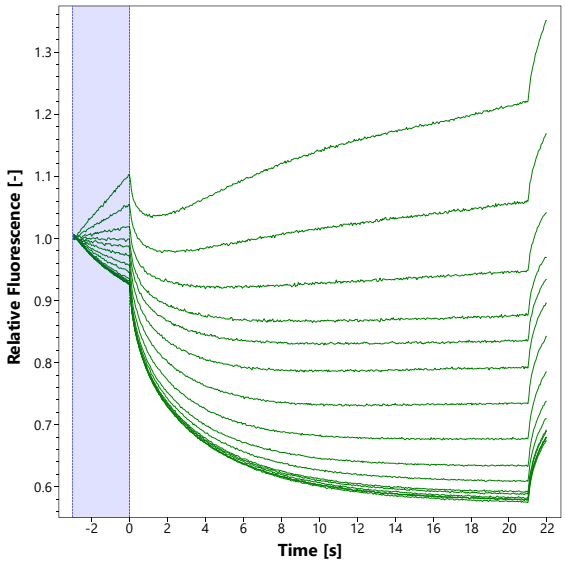
D3. MST Experiment (Assay Buffer/Concentrations/Temperature/MST Power/Excitation Power)

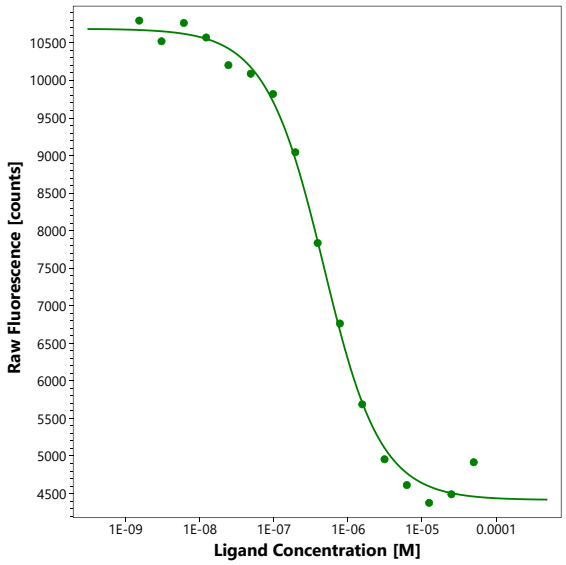
50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 0.1% Pluronic® F-127, 0.5% DMSO
200 nM bCA-II | 50 µM – 1.5 nM furosemide | 20°C | medium MST power | 60% excitation power

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

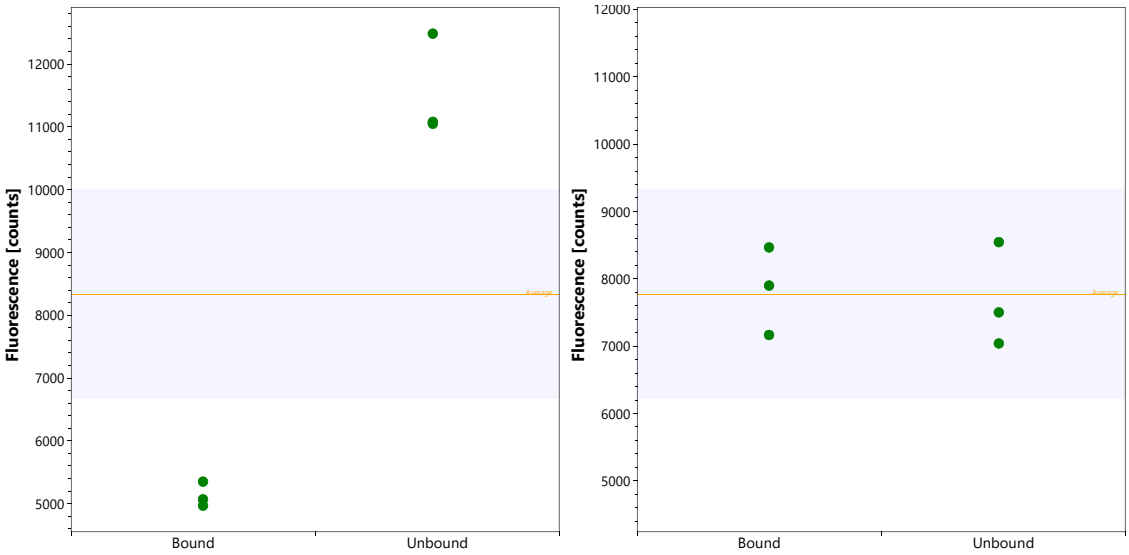
K_d = 366 nM



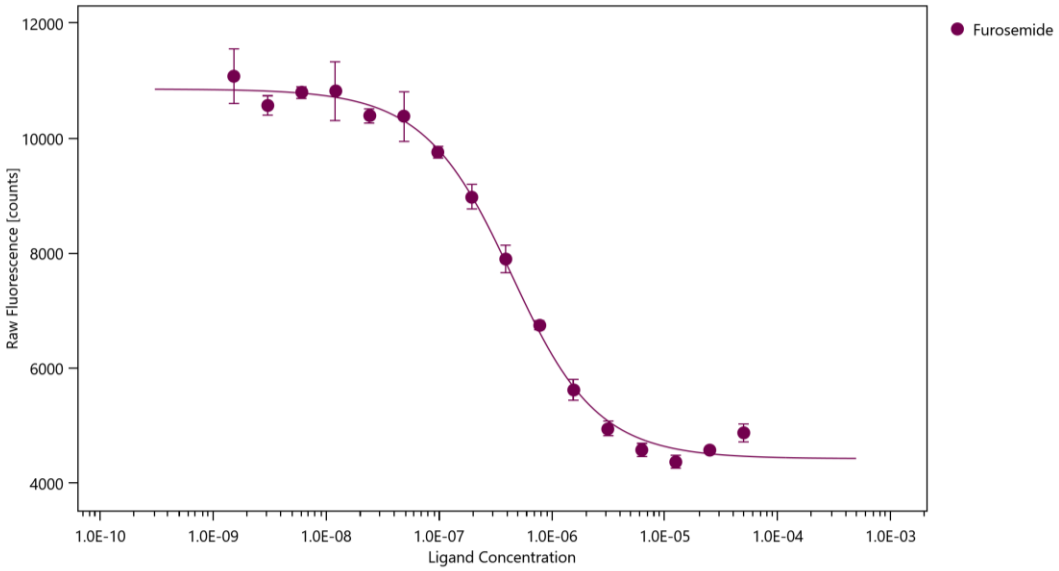




SD-Test[1]:



K_d = 336 ± 78 nM (n = 3) | S/N = 36.2



D5. Reference Results/Supporting Results

K_d = 513 nM Surface Plasmon Resonance (SPR)
Myszka et al., Analytical Biochemistry 329 (2004) 316-323

K_d = 360 nM Isothermal Titration Calorimetry (ITC)
MicroCal PEAQ-ITC, Malvern Panalytical

[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 K_d determination.

E. Contributors

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