Monolith Protocol MO-P-006

Carbonic Anhydrase II – Furosemide

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

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

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. Prepare 10 mL of Labeling Buffer NHS containing 0.1% Pluronic® F-127 ('Labeling Buffer').
2. Dissolve ~200 µg of bCA-II in 100 µL of Labeling Buffer (approx. ~70 µM concentration).
3. 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.
4. 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 Labeling Buffer.
5. Dissolve 0.34 g of $ZnCl_2$ in 10 mL of ddH_2O to obtain a 250 mM $ZnCl_2$ stock solution. Add 4 drops of HCl (1.2 M) to acidify the solution and completely dissolve all $ZnCl_2$.
6. Mix 4 µL of the 250 mM $ZnCl_2$ stock with 496 µL of ddH_2O to obtain a 500 µL of a 1 mM $ZnCl_2$ solution[1].
7. Mix 2 µL of the 1 mM $ZnCl_2$ solution with 48 µL of Labeling Buffer. Then, add 50 µL of 40 µM bCA-II to obtain 100 µL of a 20 µM bCA-II, 20 µM $ZnCl_2$ solution.
8. Add 25 µL of DMSO to 10 µg 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.
9. Mix 10 µL of the 600 µM dye solution with 90 µL of Labeling Buffer to obtain 100 µL of a 60 µM dye solution (3x protein concentration).
10. Mix bCA-II and dye in a 1:1 volume ratio (200 µL final volume, 5% final DMSO concentration).
11. Incubate for 30 minutes at room temperature in the dark.
12. 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.
13. 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.
14. Add 200 µL of the labeling reaction from step 5 to the center of the column and let sample enter the bed completely.
15. Add 450 µL of dilution buffer after the sample has entered and discard the flow through[2].
16. Place column in a new collection tube, add 450 µL of dilution buffer and collect the eluate.
17. Centrifuge the tube at 15,000 rpm at 4°C for 20 minutes to remove aggregates. Then, carefully transfer 400 µL of the supernatant of the tube into a new tube and mix well by pipetting.
18. Keep the labeled bCA-II (~3.2 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

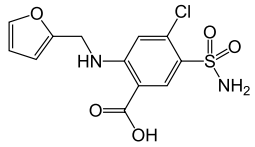
Absorbance A_{280}	0.182	Protein concentration	3.2 µM
Absorbance A_{650}	0.558	Degree-of-labeling (DOL)	0.90

[1] Due to the low solubility of $ZnCl_2$ at non-acidic pH and higher ionic strength, as well as its tendency to form insoluble $Zn(OH)_2$ at pH > 7, the $ZnCl_2$ stock is prepared in ddH_2O (see also Krezel et al., Archives of Biochemistry and Biophysics 611 (2016) 3-19).

[2] This step will also remove any excess Zn(II) ions from the sample.

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

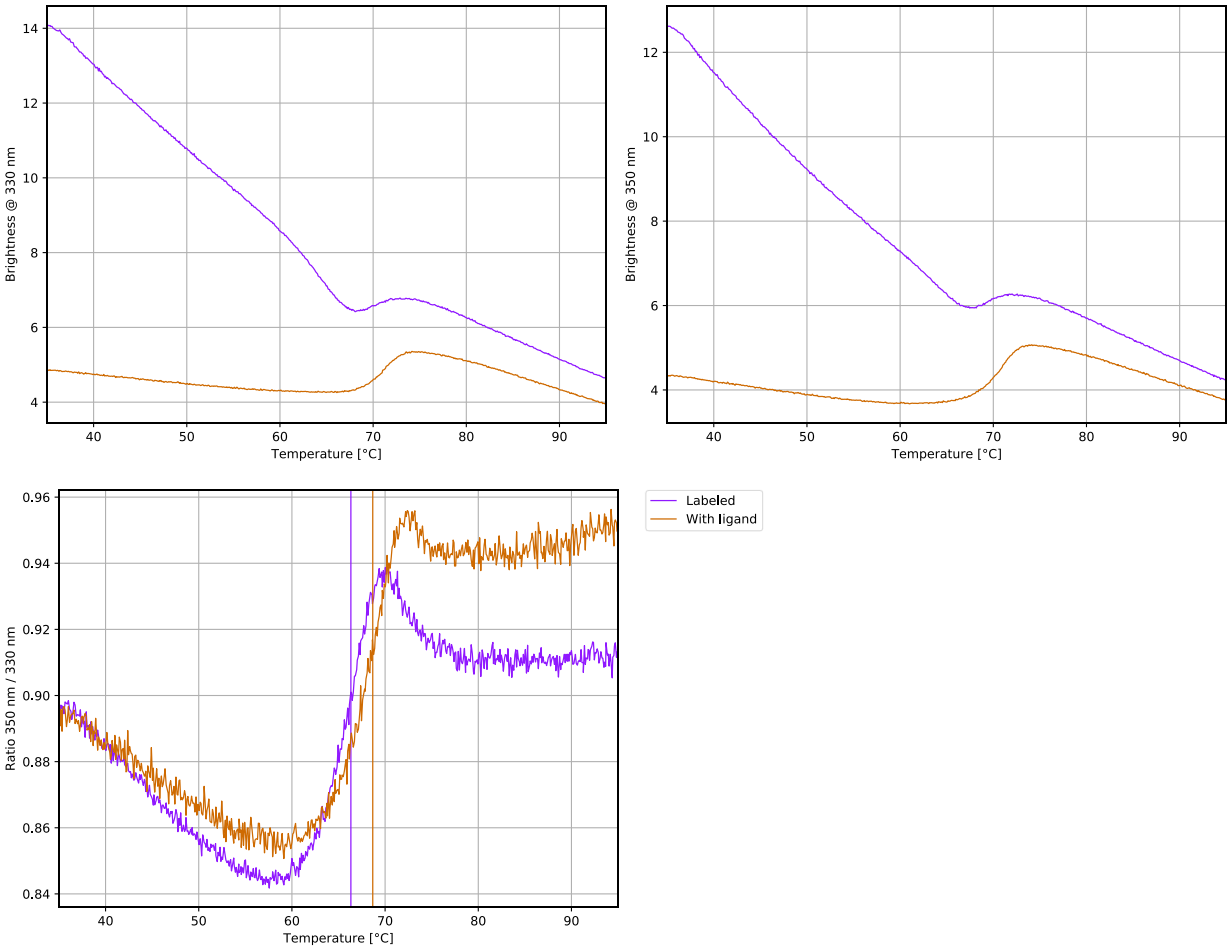
B7. Serial Dilution Preparation

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 100 µL of the 100 µM furosemide solution into tube **1**. Then, transfer 50 µL of the 1% DMSO solution into tubes **2** to **16**.
6. Prepare a 1:1 serial dilution by transferring 50 µL from tube to tube. Mix carefully by pipetting up and down. Remember to discard 50 µL from tube **16** to get an equal volume of 50 µL for all samples.
7. Mix 6 µL of labeled bCA-II (~3.2 µM) with 954 µL of dilution buffer to obtain 960 µL of ~20 nM bCA-II.
8. Add 50 µL of labeled bCA-II (~20 nM) to each tube from **16** to **1** and mix by pipetting.
9. Incubate tubes for 5 minutes in the dark before loading capillaries.

C. Applied Quality Checks

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

Labeled	5 µL of column B eluate (~3.2 µM) + 5 µL of dilution buffer containing 1% DMSO	T_i = 66.3°C
With ligand	5 µL of column B eluate (~3.2 µM) + 5 µL of 100 µM furosemide	T_i = 68.7°C



D1. MST System/Capillaries

Monolith Pico – RED (NanoTemper Technologies GmbH)
Premium Capillaries Monolith NT.115 (MO-K025, NanoTemper Technologies GmbH)

D2. MST Software

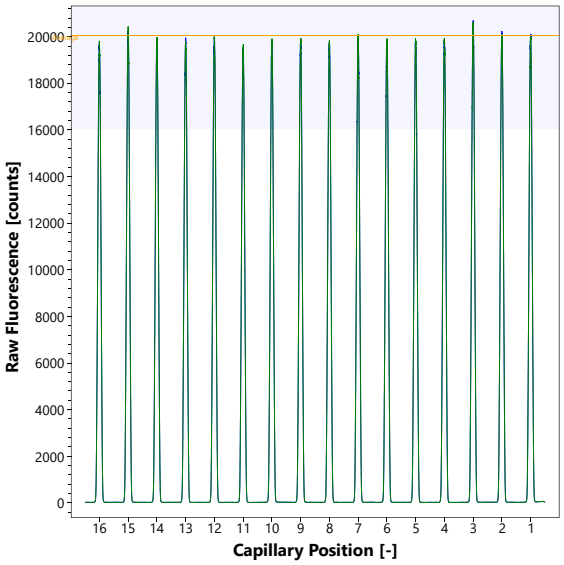
MO.Control v2.0.4 (NanoTemper Technologies GmbH)
nanotempertech.com/monolith-mo-control-software

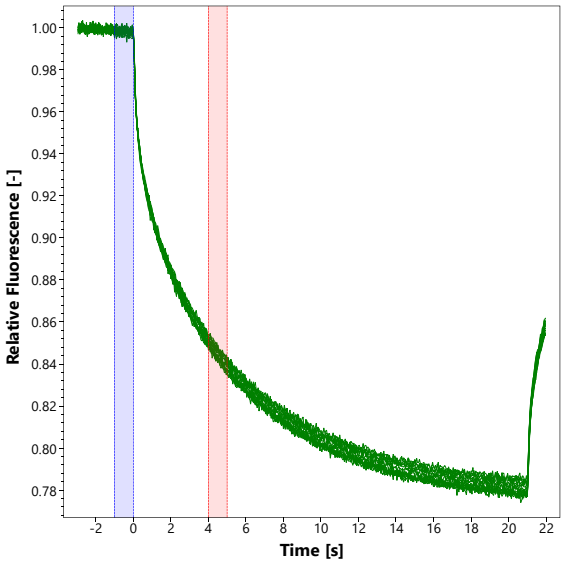
D3. MST Experiment (Assay Buffer/Concentrations/Temperature/MST Power/Excitation Power)

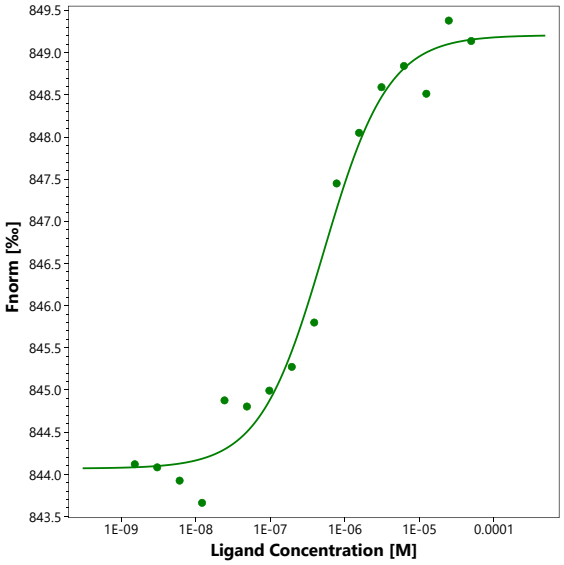
50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 0.1% Pluronic® F-127, 0.5% DMSO
10 nM bCA-II | 50 µM – 1.5 nM furosemide | 20°C | medium MST power | 10% excitation power

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

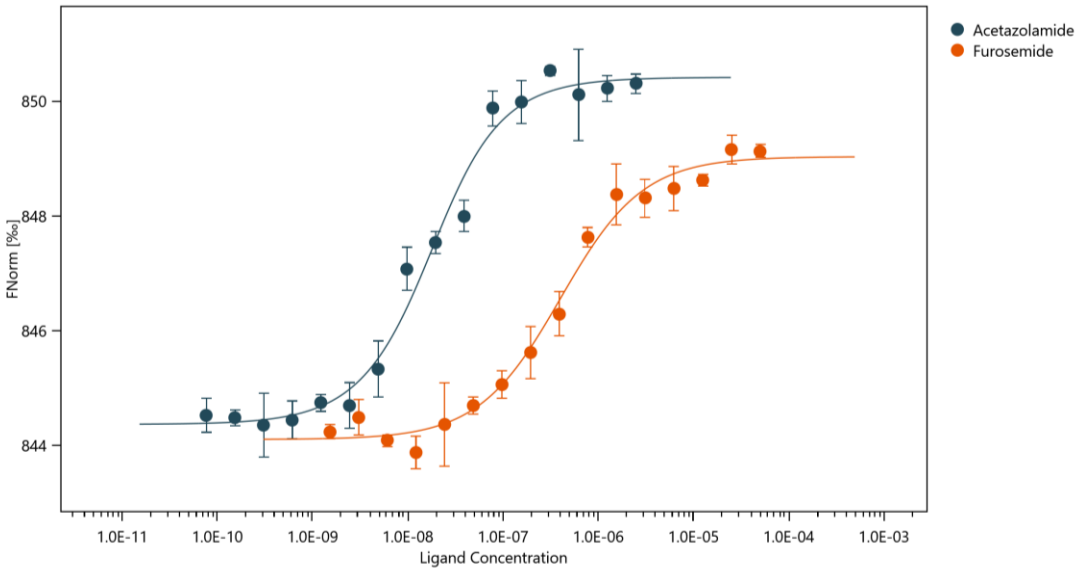
K_d = 519 nM







K_d = 398 ± 136 nM (n = 3) | S/N = 22.0



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

E. Contributors

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