

Monolith Protocol M0-P-005

Carbonic Anhydrase II – Acetazolamide

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

MSHHWGYGKH NGPEHWHKDF PIANGERQSP VDIDTKAVVQ DPALKPLALV YGEATSRRMV NNGHSFNVEY DDSQDKAVLK
DGPLTGTYRL VQFHFHWGSS DDQGSEHTVD RKKYAAELHL VHWNTKYGDF GTAAQQPDGL AVVGVLKVG DANPALQKVL
DALDSIKTKG KSTDFPNFDP GSLLPNVLDY WTPGSLTTP PLLESVTWIV LKEPISVSSQ QMLKFRTLNF NAEGEPELLM
LANWRPAQPL KNRQVRGFPK

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⁻¹cm⁻¹ (ε₂₈₀)

A7. Dilution Buffer

50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM MgCl₂, 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⁻¹cm⁻¹ (ε₂₈₀). Afterwards, adjust the concentration to 40 µM with Labeling Buffer.
5. Dissolve 0.34 g of ZnCl₂ in 10 mL of ddH₂O to obtain a 250 mM ZnCl₂ stock solution. Add 4 drops of HCl (1.2 M) to acidify the solution and completely dissolve all ZnCl₂.
6. Mix 4 µL of the 250 mM ZnCl₂ stock with 496 µL of ddH₂O to obtain a 500 µL of a 1 mM ZnCl₂ solution¹.
7. Mix 2 µL of the 1 mM ZnCl₂ 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 ZnCl₂ 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 through².
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

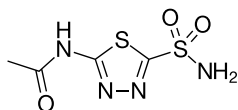
Absorbance A ₂₈₀	0.182	Protein concentration	3.2 µM
Absorbance A ₆₅₀	0.558	Degree-of-labeling (DOL)	0.90

¹ Due to the low solubility of ZnCl₂ at non-acidic pH and higher ionic strength, as well as its tendency to form insoluble Zn(OH)₂ at pH > 7, the ZnCl₂ stock is prepared in ddH₂O (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

Acetazolamide



B2. Molecule Class/Organism

Carbonic anhydrase inhibitor

B3. Sequence/Formula

$C_4H_6N_4O_3S_2$

B4. Purification Strategy/Source

Sigma-Aldrich GmbH

A6011

B5. Stock Concentration/Stock Buffer

22.2 mg/mL | 100 mM

DMSO

B6. Molecular Weight/Extinction Coefficient

222.25 Da

B7. Serial Dilution Preparation

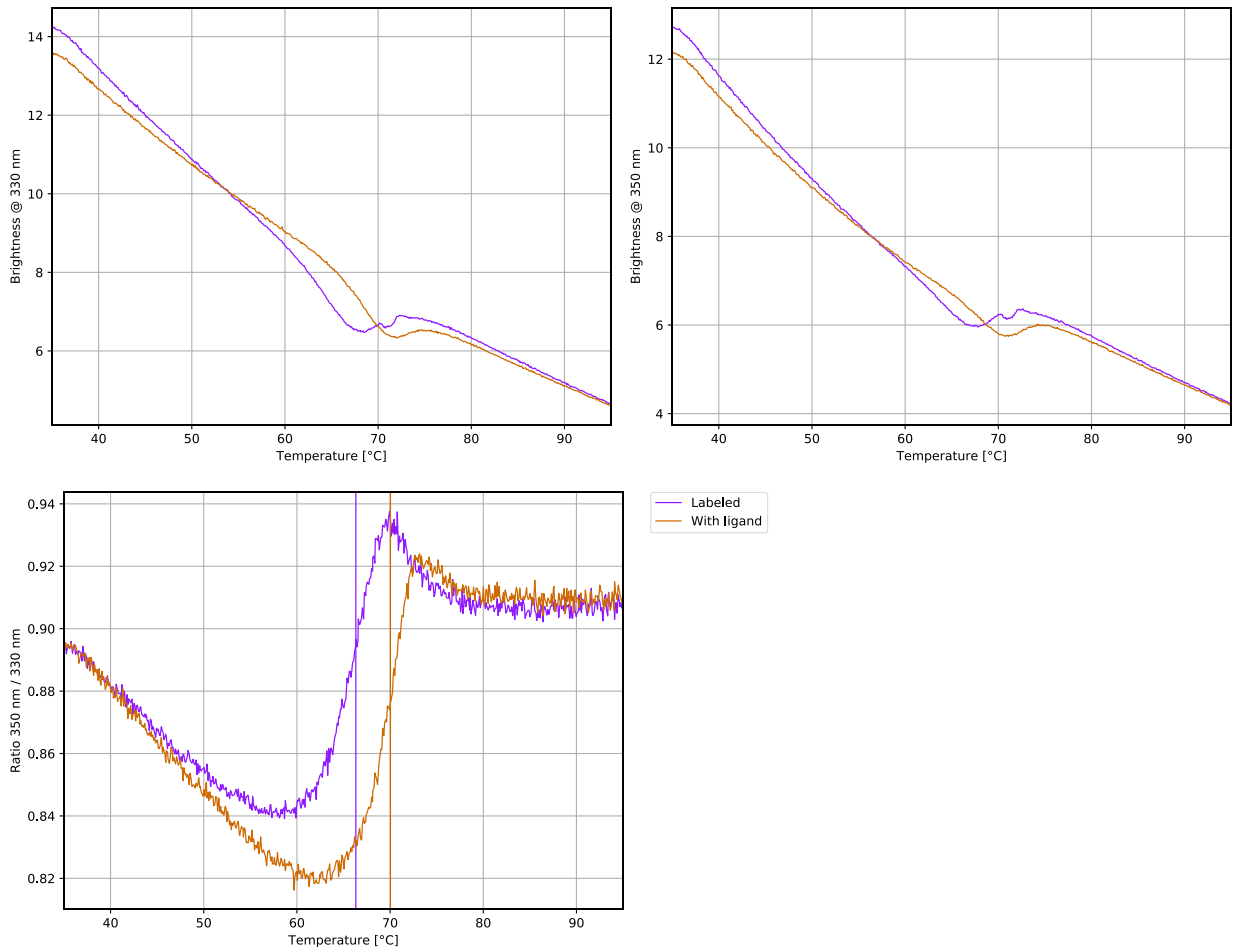
1. Dissolve 22.2 mg of acetazolamide in 1 mL of DMSO to obtain a 100 mM acetazolamide solution.
2. Add 2 μ L of 100 mM acetazolamide to 198 μ L of DMSO to obtain 200 μ L of a 1 mM solution.
3. Mix 5 μ L of the 1 mM acetazolamide solution with 495 μ L of dilution buffer to obtain 500 μ L of a 10 μ M acetazolamide solution.
4. Mix 10 μ L of DMSO with 990 μ L of dilution buffer to obtain 1 mL of a 1% DMSO solution.
5. Prepare a PCR-rack with 16 PCR tubes. Transfer 50 μ L of the 1% DMSO solution into tubes **1** to **16**. Then, transfer 50 μ L of the 10 μ M acetazolamide solution into tube **1**.
6. Prepare a 1:1 serial dilution by transferring 50 μ L from tube to tube. Mix carefully by pipetting up and down. Remember to discard 50 μ L from tube **16** to get an equal volume of 50 μ L for all samples.
7. Mix 6 μ L of labeled bCA-II (~3.2 μ M) with 954 μ L of dilution buffer to obtain 960 μ L of ~20 nM bCA-II.
8. Add 50 μ L 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 μ L of column B eluate ($\sim 3.2 \mu$ M) + 5 μ L of dilution buffer containing 1% DMSO	$T_i = 66.3^\circ\text{C}$
With ligand	5 μ L of column B eluate ($\sim 3.2 \mu$ M) + 5 μ L of 10 μ M acetazolamide	$T_i = 70.0^\circ\text{C}$



D1. MST System/Capillaries

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

D2. MST Software

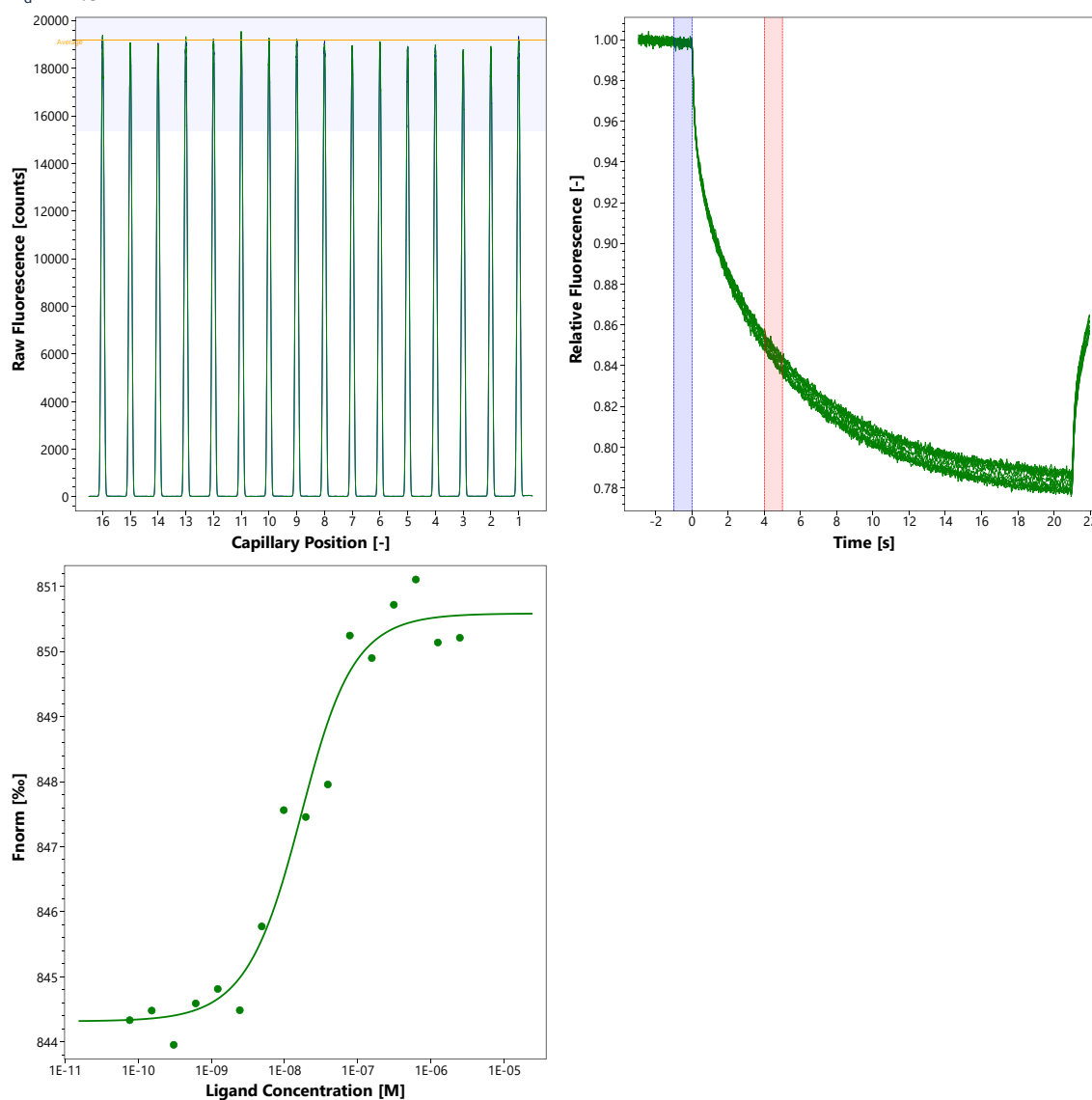
MO.Control v2.0.4 (NanoTemper Technologies GmbH)
nanotempertech.com/monolith-mo-control-software

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

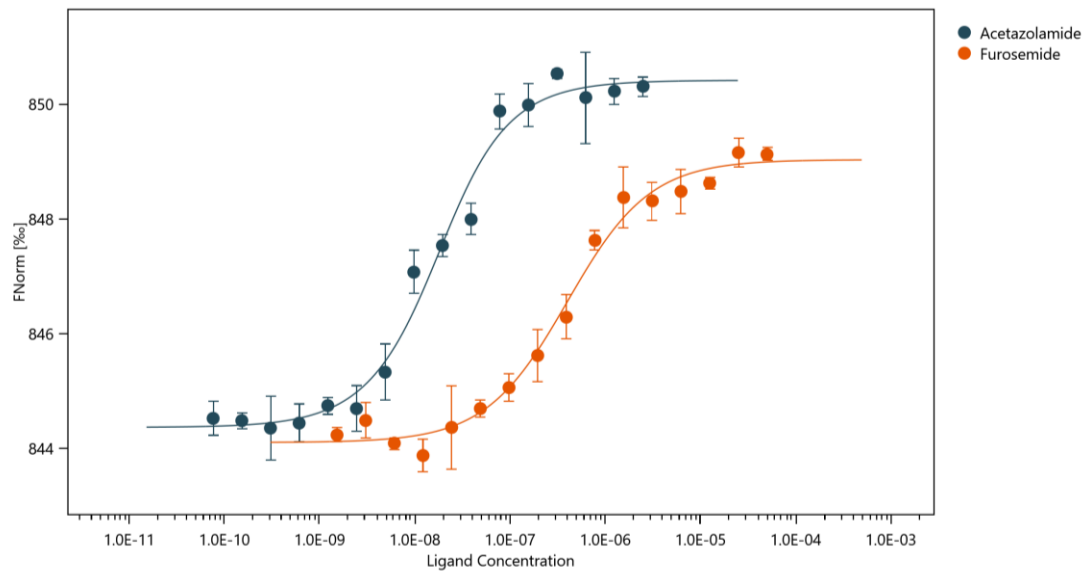
50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM MgCl₂, 0.1% Pluronic® F-127, 0.5% DMSO
10 nM bCA-II | 2.5 μ M – 76.3 pM acetazolamide | 20°C | medium MST power | 10% excitation power

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

$K_d = 11.5$ nM



$K_d = 12.7 \pm 6.3 \text{ nM}$ (n = 3) | S/N = 19.4



D5. Reference Results/Supporting Results

$K_d = 19 \text{ nM}$	Surface Plasmon Resonance (SPR) Myszka et al., Analytical Biochemistry 329 (2004) 316-323
$K_d = 18 \text{ nM}$	Isothermal Titration Calorimetry (ITC) MicroCal PEAQ-ITC, Malvern Panalytical
$K_d = 41 \pm 10 \text{ nM}$	MicroScale Thermophoresis (MST) Rogez-Florent et al., Journal of Pharmaceutical and Biomedical Analysis 137 (2017) 113-122

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