Monolith X Protocol MOX-P-110

Lysozyme – NAG_3

Lysozyme is an enzyme that prevents bacterial infections by attacking peptidoglycan, a component of certain bacterial cell walls. Peptidoglycan is composed of the repeating amino sugars N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), which are crosslinked by peptide bridges. Lysozyme hydrolyzes the bond between NAG and NAM, increasing the bacteria's permeability and causing the bacteria to burst. It is widely distributed in plants and animals. Most of the lysozyme used in research is purified from hen egg whites. Tri-N-acetyl-D-glucosamine (NAG_3) is an inhibitor of lysozyme.

protein – small molecule | carbohydrate

A1. Target/Fluorescent Molecule

Lysozyme
uniprot.org/uniprot/P00698

A2. Molecule Class/Organism

Glycoside hydrolase
Gallus gallus (Chicken)

A3. Sequence/Formula

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KVFGRCELAA AMKRHGLDNY RGYSLGNWVC AAKFESNFNT QATNRNTDGS TDYGILQINS RWWCNDGRTP GSRNLCNIPC
SALLSSDITA SVNCAKKIVS DGNGMNAWVA WRNRCKGTDV QAWIRGCRL
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A4. Purification Strategy/Source

Sigma-Aldrich GmbH
62970

A5. Stock Concentration/Stock Buffer

32 µg lyophilized powder

A6. Molecular Weight/Extinction Coefficient

14.3 kDa
37,970 $M^{-1}cm^{-1}$ (ε_{280})

A7. Dilution Buffer

Phosphate-buffered saline (PBS), pH 7.4, 0.01% Pluronic® F-127

A8. Labeling Strategy

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

A9. Labeling Procedure

1. Resuspend 32 µg lysozyme in 112 µL of dilution buffer to obtain a 20 µM lysozyme solution.
2. Transfer 80 µL of the 20 µM lysozyme solution into a new tube.
3. Add 25 µL of DMSO to 10 µg RED-NHS 2nd Generation dye to obtain a ~600 µM solution. Mix the dye thoroughly by vortexing and make sure that all dye is dissolved.
4. Mix 8 µL of the 600 µM dye solution with 72 µL of dilution buffer[1] to obtain 80 µL of a 60 µM dye solution (3x protein concentration).
5. Mix lysozyme and dye in a 1:1 volume ratio (160 µL final volume, ~5% 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 160 µL of the labeling reaction from step 5 to the center of the column and let sample enter the resin bed completely.
10. Add 600 µL of dilution buffer after the sample has entered and discard the flow through.
11. Place column in a new collection tube, add 400 µL of dilution buffer and collect the eluate.
12. Centrifuge the eluate at 15,000 rpm and 4°C for 30 minutes to remove aggregates. Then, carefully transfer the supernatant into a fresh tube and mix well by pipetting. Make sure not to disturb the blue pellet at the bottom of the tube with the pipette tip.
13. Prepare 10 µL aliquots of the labeled Lysozyme (~5 µM) and store at -80°C.

A10. Labeling Efficiency

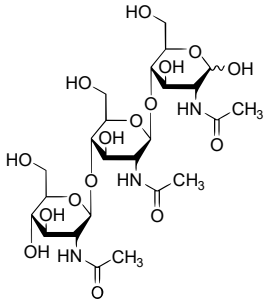
Measurement of protein concentration and degree of labeling (DOL) using a NanoDrop™:
nanotempertech.com/dol-calculator

Absorbance A_{280}	0.089	Protein concentration	~2.04 µM
Absorbance A_{650}	0.284	Degree-of-labeling (DOL)	~0.71

[1] Do not use Labeling Buffer NHS for labeling as lysozyme tends to aggregate at alkaline pH.

B1. Ligand/Non-Fluorescent Binding Partner

Tri-N-acetyl-D-glucosamine (NAG_3)



B2. Molecule Class/Organism

Carbohydrate

B3. Sequence/Formula

$C_{24}H_{41}N_3O_{16}$

B4. Purification Strategy/Source

Sigma-Aldrich GmbH
T2144

B5. Stock Concentration/Stock Buffer

25 µg powder

B6. Molecular Weight/Extinction Coefficient

627.59 Da

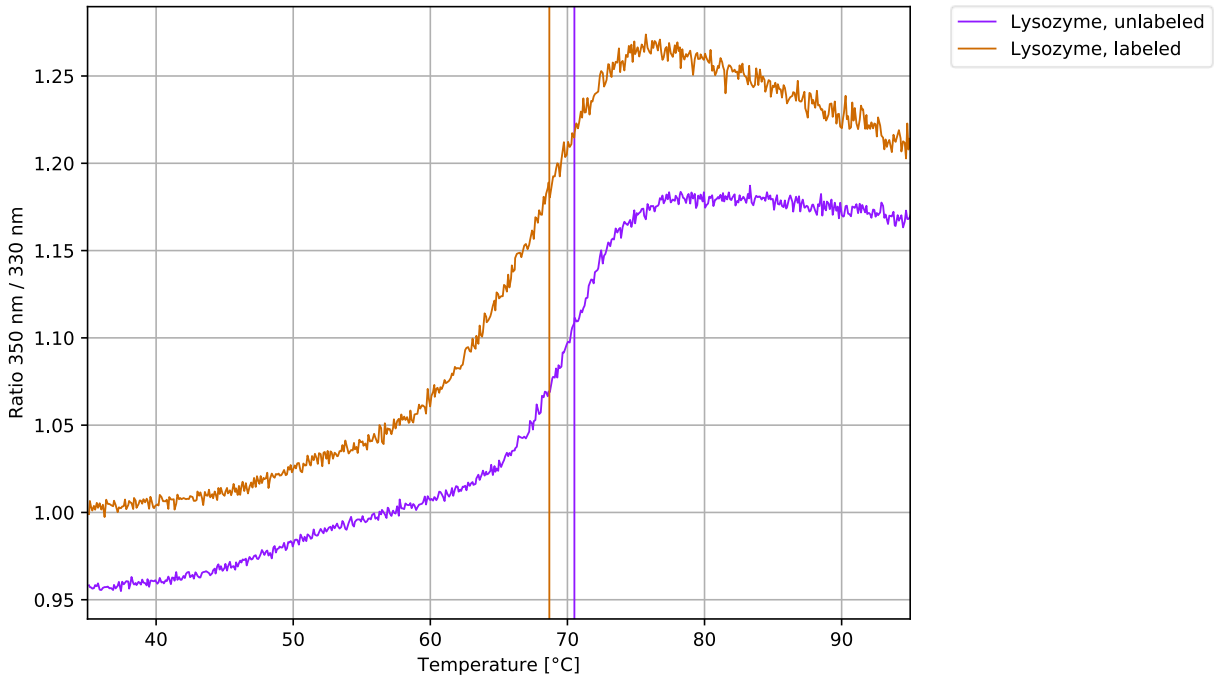
B7. Serial Dilution Preparation

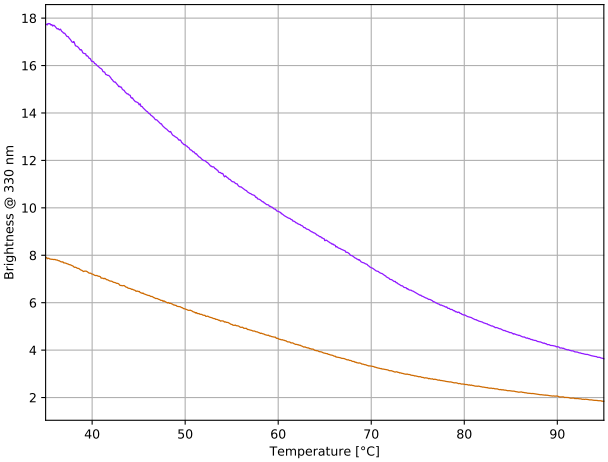
1. Resuspend NAG_3 in 25 µL of dilution buffer to obtain a 1.6 mM solution.
2. Mix 4 µL of labeled lysozyme (~5 µM) with 196 µL of dilution buffer to obtain 200 µL of a ~100 nM lysozyme solution.
3. Take a fresh 0.5 mL tube and mix 160 µL of 100 nM lysozyme with 160 µL of dilution buffer to obtain 320 µL of a 50 nM lysozyme solution.
4. Prepare a PCR-rack with 16 PCR tubes. Transfer 20 µL of the 50 nM lysozyme solution into tubes **2** to **16**. Then, mix 20 µL of 1.6 mM NAG_3 with 20 µL of 100 nM lysozyme in tube **1**.
5. Prepare a 1:1 serial dilution by transferring 20 µL from tube to tube. Mix carefully by pipetting up and down.
6. Load capillaries immediately.

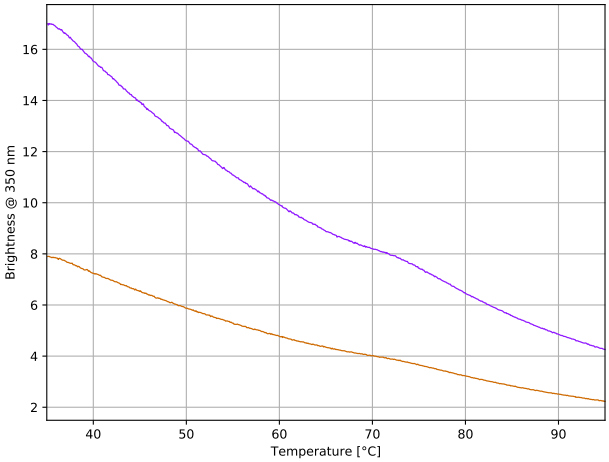
C. Tycho

Validation of structural integrity of labeled lysozyme using Tycho NT.6:
nanotempertech.com/tycho

Lysozyme, unlabeled	2.5 µL of 20 µM lysozyme + 7.5 µL of dilution buffer	T_i = 70.5°C
Lysozyme, labeled	10 µL of B-Column eluate (~5 µM)	T_i = 68.7°C



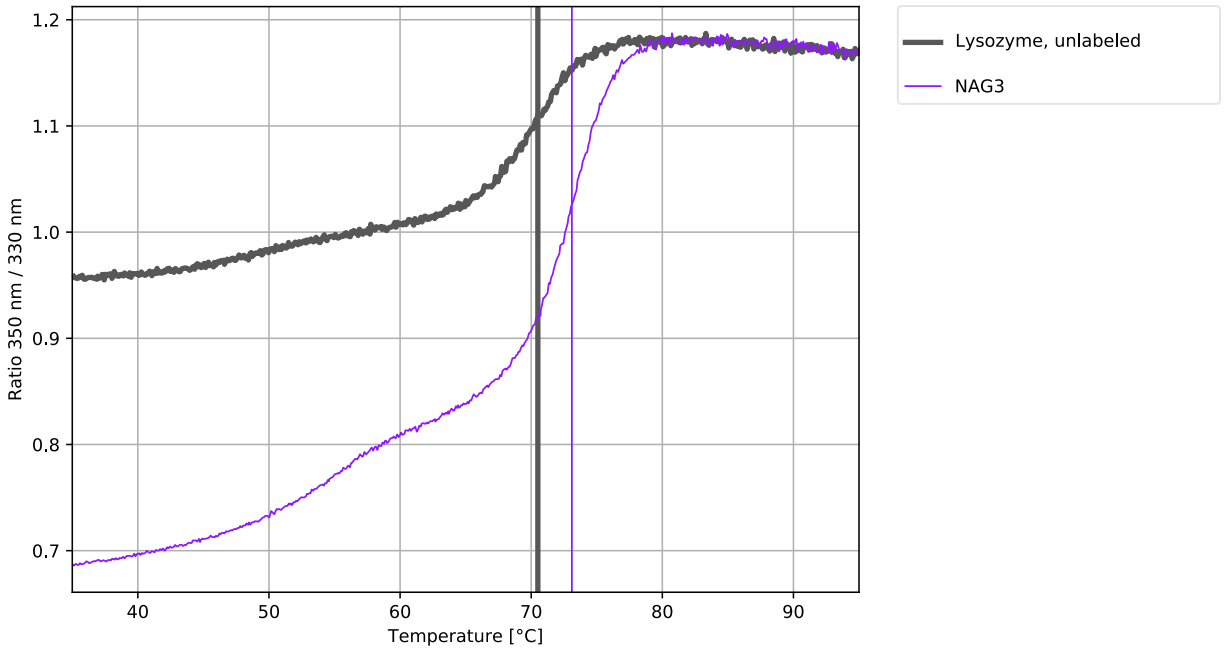


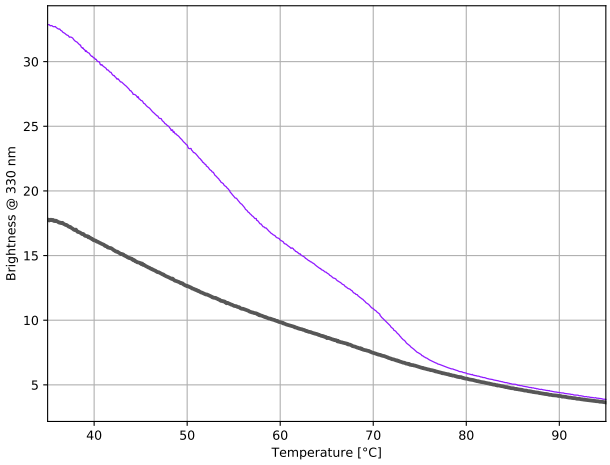


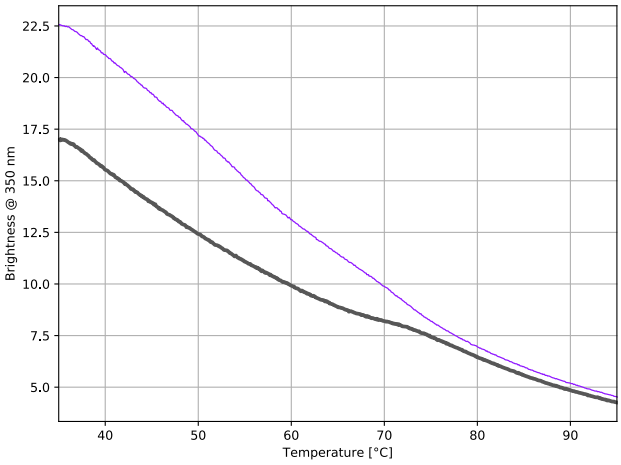
Validation of functionality of lysozyme using Tycho NT.6:

nanotempertech.com/tycho

NAG_3	2.5 µL of 20 µM lysozyme + 2.5 µL of dilution buffer + 5 µL of 1.6 mM NAG_3	T_i = 73.1°C







D1. Monolith System/Capillaries

Monolith X (NanoTemper Technologies GmbH)
Monolith Premium Capillaries (MO-K025, NanoTemper Technologies GmbH)

D2. Monolith Software

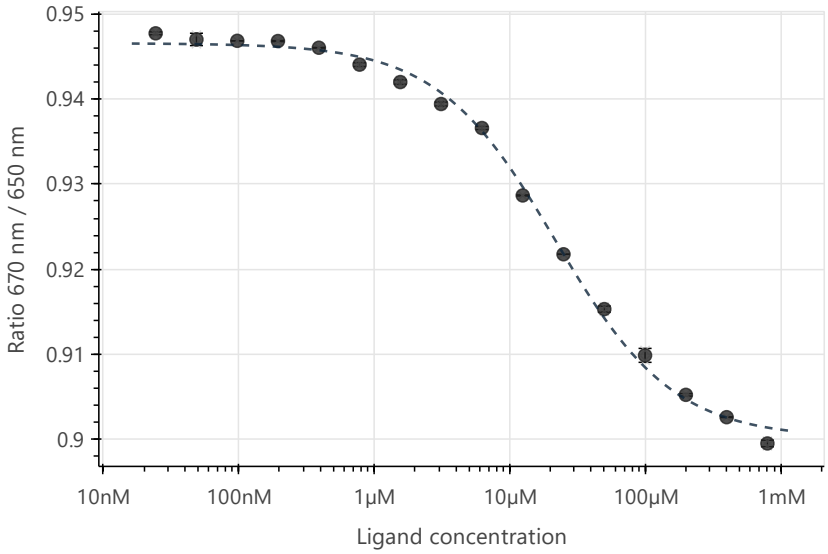
MO.Control v2.4.2 (NanoTemper Technologies GmbH)
nanotempertech.com/monolith-mo-control-software

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

Phosphate-buffered saline (PBS), pH 7.4, 0.01% Pluronic® F-127
50 nM lysozyme | 800 µM – 24 nM NAG_3 | 20°C | 50% excitation power

D4. Monolith Results (Dose Response)

K_d = 27.9 ± 2.1 µM (S/N = 44.4)



D5. Reference Results/Supporting Results

K_d = 8.2 µM	Isothermal Titration Calorimetry (ITC) Wei et al., J. Chem. Educ. 92, 9 (2015) 1552–1556
K_d = 39.8 µM	NanoESI Jecklin et al., J Am Soc Mass Spectrom 19 (2008) 332–343
K_d = 39 µM	Microchip-ESI-MS Svobodova et al., Electrophoresis 31 (2010) 2680–2685

E. Contributors

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