Monolith Protocol MO-P-010

Lysozyme – NAG_3 (biotinylated)

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. The majority of the lysozyme used in research is purified from hen egg whites. Tri-N-acetyl-D-glucosamine (NAG_3) is an inhibitor of lysozyme. The interaction of biotinylated lysozyme with NAG_3 can be measured using fluorescently labeled streptavidin.

protein – small molecule interaction | carbohydrate | biotinylation | streptavidin

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A1. Target/Fluorescent Molecule

Lysozyme (biotinylated)
uniprot.org/uniprot/B8YK79

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

N/A

A5. Stock Concentration/Stock Buffer

48 µg/ml | 3.4 µM
Phosphate-buffered saline (PBS), pH 7.4

A6. Molecular Weight/Extinction Coefficient

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

A7. Dilution Buffer

50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% TWEEN® 20

A8. Labeling Strategy

Labeling of biotinylated lysozyme via binding to labeled streptavidin

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 100 µL of a 10 µM streptavidin (52.8 kDa, 167,000 $M^{-1}cm^{-1}$ (ε_{280})) solution in Labeling Buffer NHS.
2. 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.
3. Mix 10 µL of the 600 µM dye solution with 90 µL of Labeling Buffer NHS to obtain 100 µL of a 60 µM dye solution (6x protein concentration).
4. Mix streptavidin and dye in a 1:1 volume ratio (200 µL final volume, 5% final DMSO concentration).
5. Incubate for 30 minutes at room temperature in the dark.
6. 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.
7. 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.
8. Add 200 µL of the labeling reaction from step 5 to the center of the column and let sample enter the resin bed completely.
9. Add 400 µL of dilution buffer after the sample has entered and discard the flow through.
10. Place column in a new collection tube, add 500 µL of dilution buffer and collect the eluate.
11. Mix 2 µL of labeled streptavidin with 5.9 µL of 3.4 µM biotinylated lysozyme and 192 µL of dilution buffer to obtain 200 µL of ~20 nM streptavidin, 100 nM biotinylated lysozyme (5x streptavidin concentration)[1].
12. Keep the streptavidin-labeled lysozyme (100 nM) on ice in the dark.

A10. Labeling Efficiency

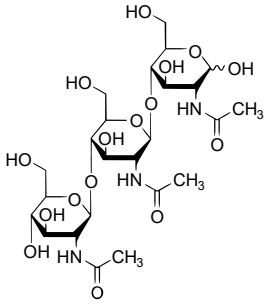
Measurement of protein concentration and degree of labeling (DOL) using a NanoDrop™:
nanotempertech.com/dol-calculator

Absorbance A_{280}	0.394	Protein concentration	~2.20 µM
Absorbance A_{650}	0.650	Degree-of-labeling (DOL)	~1.53

[1] As streptavidin is a tetrameric protein and each streptavidin monomer can bind one biotin molecule, a streptavidin protein can maximally bind four biotins.

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
T2144

B5. Stock Concentration/Stock Buffer

25 µg powder

B6. Molecular Weight/Extinction Coefficient

627.59 Da

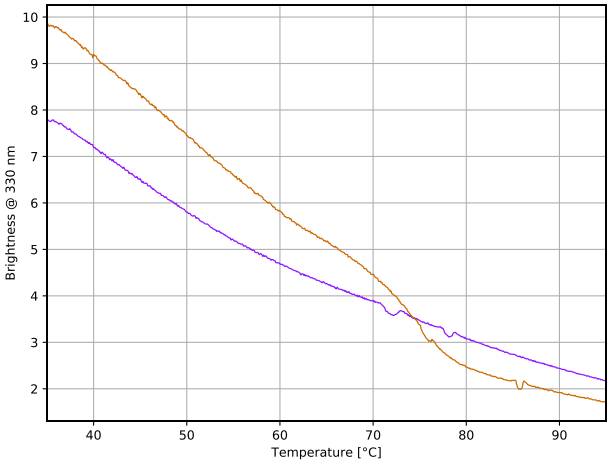
B7. Serial Dilution Preparation

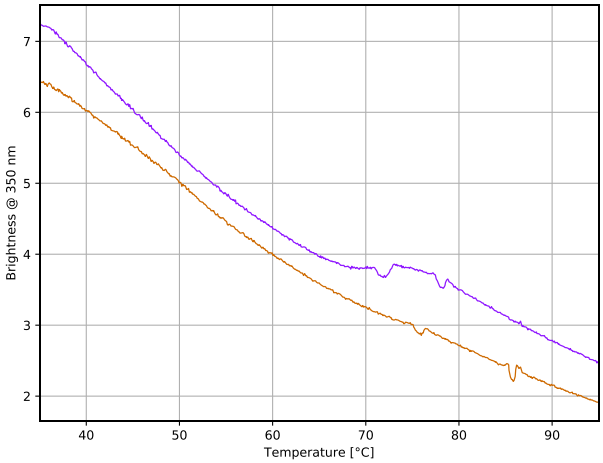
1. Resuspend NAG_3 in 25 µL of dilution buffer to obtain a 1.6 mM solution.
2. Prepare a PCR-rack with 16 PCR tubes. Transfer 20 µL of the 1.6 mM NAG_3 solution into tube **1**. Then, transfer 10 µL of dilution buffer into tubes **2** to **16**.
3. Prepare a 1:1 serial dilution by transferring 10 µL 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.
4. Add 10 µL of streptavidin-labeled lysozyme (100 nM) to each tube from **16** to **1** and mix by pipetting.
5. Incubate for 5 minutes at room temperature in the dark before loading capillaries.

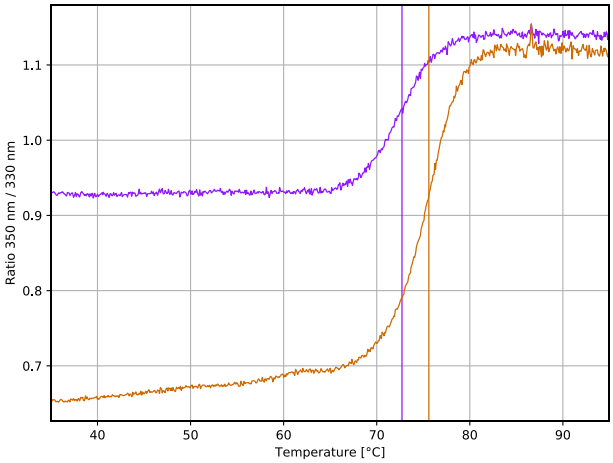
C. Applied Quality Checks

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

Unlabeled	5 µL of 3.4 µM biotinylated lysozyme + 5 µL of dilution buffer	T_i = 72.7°C
With ligand	5 µL of 3.4 µM biotinylated lysozyme + 5 µL of 1.6 mM NAG_3	T_i = 75.6°C

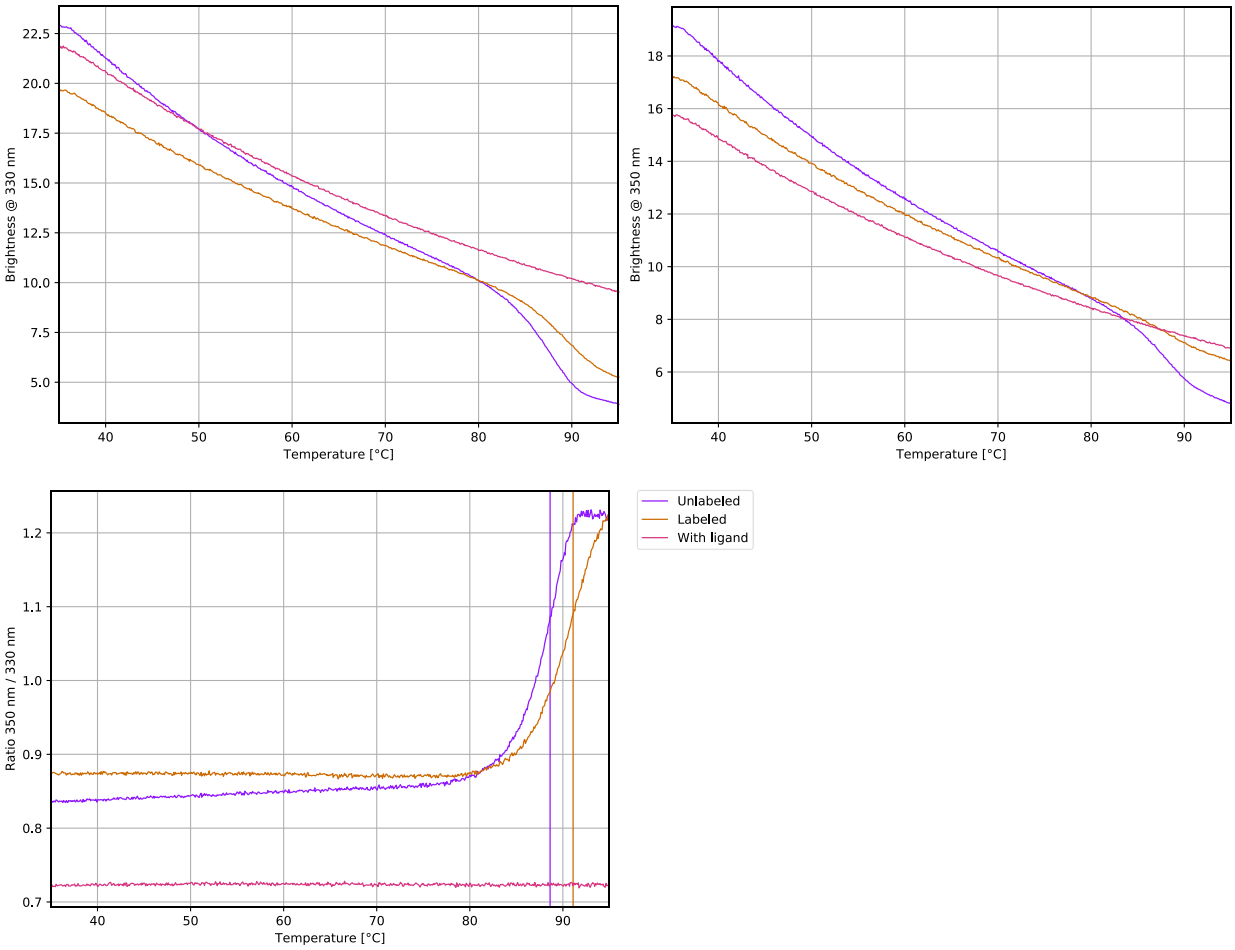






Validation of structural integrity and functionality of labeled streptavidin using Tycho NT.6:

Unlabeled	5 µL of 2 µM streptavidin + 5 µL of dilution buffer	T_i = 88.6°C
Labeled	5 µL of B-Column eluate (~2 µM) + 5 µL of dilution buffer	T_i = 91.1°C
With ligand	5 µL of B-Column eluate (~2 µM) + 5 µL of 1 mM biotin	T_i > 95°C[2]



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 or higher (NanoTemper Technologies GmbH)
nanotempertech.com/monolith-mo-control-software

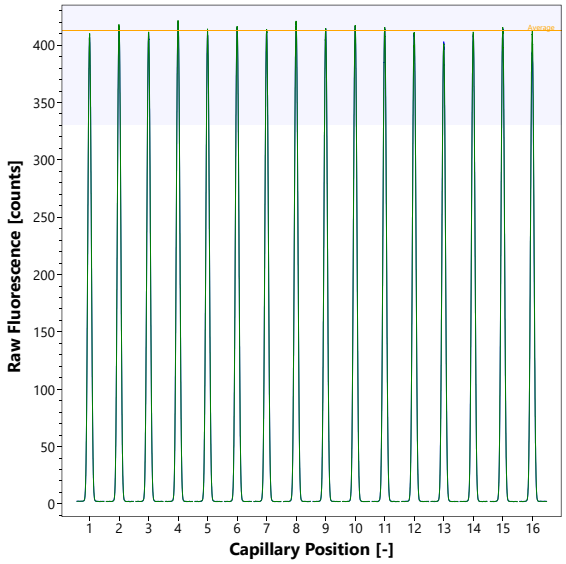
[2] Biotin has been shown to shift the T_m of streptavidin from 75°C to 112°C (González, 1997).

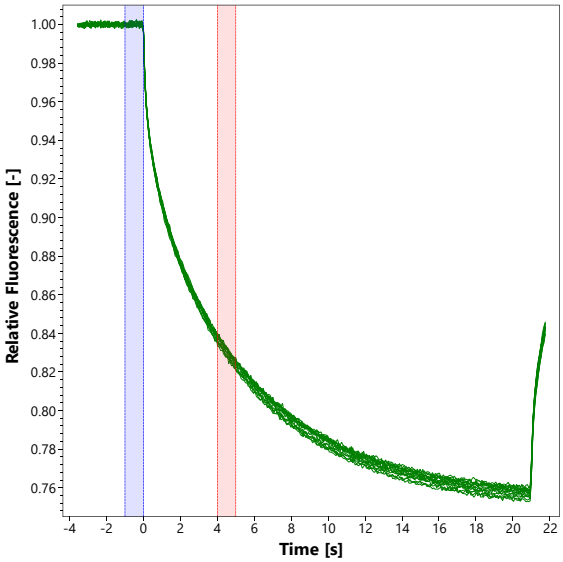
D3. MST Experiment (Assay Buffer/Concentrations/Temperature/MST Power/Excitation Power)

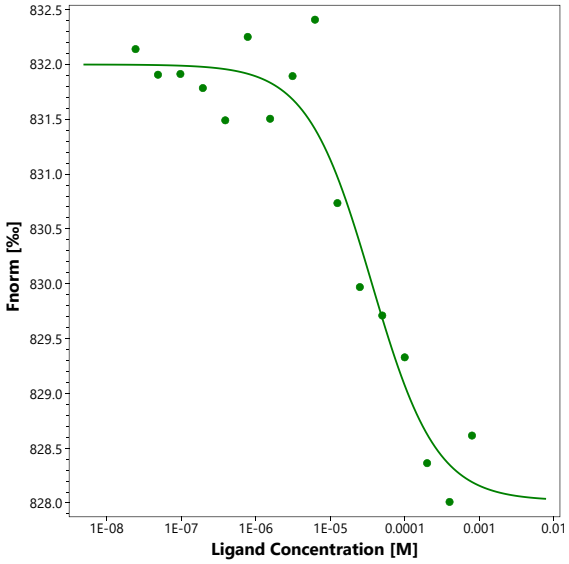
50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% TWEEN® 20
10 nM streptavidin, 50 nM lysozyme | 800 µM – 24 nM NAG_3 | 25°C | medium MST power | 40% excitation power

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

K_d = 36 µM







D5. Reference Results/Supporting Results

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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